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Date: 10/23/01 Express Mail Label No. EJ6119477 63 US

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Attorney's Docket No.: 1818.1009-009

## RED BLOOD CELLS LOADED WITH S-NITROSOTHIOL AND USES THEREFOR

### RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No.

5 09/724,305 filed on November 28, 2000, which is a continuation of U.S. Patent Application Number 08/873,679 filed on June 12, 1997 (now Patent No. 6,203,789), which is a continuation-in-part of PCT/US96/14664 filed on September 13, 1996, which is a continuation of U.S. Patent Application Number 08/616,255 filed on March 15, 1996 (now Patent No. 6,153,186), which claims the benefit of U.S. Provisional

10 10 Application Number 60/003,801 filed on September 15, 1995. The teachings of all of the above applications are each incorporated herein by reference in their entirety.

### GOVERNMENT SUPPORT

The invention was made with Government support under Grant No. HL52529 awarded by the National Institutes of Health. The Government has certain rights in the

15 invention.

### BACKGROUND OF THE INVENTION

Interactions of hemoglobin (Hb) with small diffusible ligands, such as O<sub>2</sub>, CO<sub>2</sub> and NO, are known to occur at its metal centers and amino termini. The O<sub>2</sub>/CO<sub>2</sub>

vertical margin text: PROTEIN LIGANDS OF NO

delivery functions, which arise in the lung and systemic microvasculature, are allosterically controlled. Such responsiveness to the environment has not been known to apply in the case of NO. Specifically, it has been thought previously that NO does not modify the functional properties of Hb to any physiologically significant degree.

5 Kinetic modeling predicts that the vast majority of free NO in the vasculature should be scavenged by Hb (Lancaster 1994). Accordingly, the steady-state level of NO may fall below the  $K_m$  for target enzymes such as guanylate cyclase (Lancaster 1994), if not in the unperturbed organism, then with oxidant stress such as that found in atherosclerosis. These considerations raise the fundamental question of how NO exerts its biological

10 activity.

One answer to this question is found in the propensity of nitric oxide to form S-nitrosothiols (RSNOs) (Gaston, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10957-10961 (1993)), which retain NO-like vasorelaxant activity (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)), but which can diffuse freely in and out of cells, 15 unlike Hb. In particular, the NO group of RSNOs possesses nitrosonium ( $\text{NO}^+$ ) character that distinguishes it from NO itself. It is increasingly appreciated that RSNO's have the capacity to elicit certain functions that NO is incapable of (DeGroote, M.A. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995); Stamler, J.S., *Cell* 78:931-936 (1994)). Moreover, consideration has been given to the possibility that -SNO groups in 20 proteins serve a signaling function, perhaps analogous to phosphorylation (Stamler, J.S. *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); Stamler, J.S. *Cell* 78:931-926 (1994)). Although S-nitrosylation of proteins can regulate protein function (Stamler, J.S. *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); Stamler, J.S., *Cell* 78:931-936 (1994)), the identification of S-nitrosoproteins within cells -- the *sine qua non* of a 25 regulatory posttranslational modification -- has heretofore not been demonstrated.

Hemoglobin is a tetramer comprised of two alpha and two beta subunits. In human Hb, each subunit contains one heme, while the beta ( $\beta$ ) subunits also contain highly reactive SH groups (cys $\beta$ 93) (Olson, J.S., *Meth. in Enzym.* 76:631-651 (1981);

Antonini & Brunori, *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). These cysteine residues are highly conserved among species although their function has remained elusive.

5       NO (nitric oxide) is a biological "messenger molecule" which decreases blood pressure and inhibits platelet function, among other functions. NO freely diffuses from endothelium to vascular smooth muscle and platelet and across neuronal synapses to evoke biological responses. Furthermore, under some conditions, reactions of NO with other components present in cells and in body fluids can generate toxic intermediates

10      10 and products at local concentrations in tissues which are effective at inhibiting the growth of infectious organisms. Thus, it can be seen that a method of administering an effective concentration of NO or biologically active forms thereof would be beneficial in certain medical disorders.

#### SUMMARY OF THE INVENTION

15      The invention relates to a method for the extracorporeal loading of red blood cells (RBCs) with a low molecular weight reagent which is capable of entering the red blood cells and causing S-nitrosylation at thiol groups. S-nitrosothiols such as S-nitrosocysteine, and related peptides can be used to load red blood cells, which can then be introduced into a mammal. The red blood cells thereby become carriers of NO. In

20      20 this manner, medical conditions characterized by abnormal oxygen metabolism of tissues, oxygen-related toxicity, abnormal vascular tone, abnormal red blood cell adhesion, or abnormal O<sub>2</sub> delivery by red blood cells, can be treated.

Other aspects of the invention are based on the discovery that NO in red blood cells, particularly in the form of S-nitrosothiols such as S-nitrosohemoglobin and S-nitrosoglutathione is at low levels in some diseases and medical conditions. Of particular importance is the NO:Hb ratio within red blood cells. Also of importance are the biochemical and structural properties of the red blood cell that allow for the transfer

of biologically active forms of NO from one thiol group to another, so that biologically active NO of the red blood cell can reach the cells of the vascular system to regulate blood vessel tension.

Included in the invention are methods for raising NO:hemoglobin in red blood cells in a mammal, comprising administering to the mammal a therapeutically effective amount of a composition comprising NO, a composition comprising an inorganic nitrite, a composition comprising an organic nitrite, or a composition comprising a donor of biologically active NO.

Further aspects of the invention are methods for alleviating the effects of a medical intervention in a human, said intervention resulting in a low NO:hemoglobin ratio in red blood cells, said method comprising administering to the mammal a therapeutically effective amount of a composition comprising NO, a composition comprising an inorganic nitrite, a composition comprising an organic nitrite, or a composition comprising a donor of biologically active NO. The medical intervention can comprise treatment with erythropoietin, treatment by blood transfusion, or treatment with one or more hemoglobin-based blood substitutes.

Other methods are to treat a condition in a mammal associated with a low NO:hemoglobin ratio in red blood cells, said method comprising administering to the mammal a therapeutically effective amount of a composition comprising NO, a composition comprising an inorganic nitrite, a composition comprising an organic nitrite, or a composition comprising a donor of biologically active NO. The condition is systemic hypertension, pulmonary hypertension, stroke, myocardial infarction, sickle cell disease, sepsis, thalassemia, polycythemia, a congenital disorder of red blood cells, coronary disease, or a hypoxic condition. Increased coronary blood flow is a consequence of the method.

Another therapeutic method involves the use of an ex vivo treatment of isolated blood, such as the incubation of the blood with NO or a donor of biologically active NO, such that the biologically active NO, especially in the form of S-nitrosothiol, is

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increased in the red blood cells. Part of the invention is a method for increasing biologically active NO in isolated red blood cells, comprising contacting the isolated red blood cells with a composition comprising a reagent selected from the group consisting of: NO, an S-nitrosothiol, an ester of an S-nitrosothiol, and ethyl nitrite. The S-nitrosothiol can be S-nitrosoglutathione, for example.

One method for raising NO:hemoglobin in red blood cells in a mammal, comprises administering to the mammal a therapeutically effective amount of a composition comprising red blood cells enriched with biologically active NO by use of an *ex vivo* treatment. The effect of such treatment will be to alleviate the effects of certain medical interventions. Thus, the invention is also a method for alleviating the effects of a medical intervention in a human, said medical intervention resulting in a low NO:hemoglobin ratio in red blood cells, said method comprising administering to the human a therapeutically effective amount of a composition comprising red blood cells enriched with biologically active NO. The medical intervention can be treatment with erythropoietin, treatment by blood transfusion, or treatment with one or more hemoglobin-based blood substitutes. The invention is in another aspect a method for treating a condition in a mammal associated with a low NO:hemoglobin ratio in red blood cells, said method comprising administering to the human a therapeutically effective amount of a composition comprising red blood cells enriched with biologically active NO. The condition can be systemic hypertension, pulmonary hypertension, stroke, myocardial infarction, sickle cell disease, sepsis, thalassemia, polycythemia, a congenital disorder of red blood cells, coronary disease, or a hypoxic condition. One effect of the method is to increase coronary blood flow.

By another method, one can potentiate NO-mediated bioactivity of red blood cells by a step or steps comprising administering to the mammal a therapeutically effective amount of a composition comprising a thiol. The thiol can be glutathione.

Methods of the invention can be applied to the blood of blood banks to be used in whole blood transfusions. One method is intended to restore to a desirable value or range of values the NO:Hb ratio, in blood for transfusion into a human, the method comprising contacting the blood with a solution comprising a reagent which can be one or more of NO, an S-nitrosothiol, an ester of an S-nitrosothiol, and ethyl nitrite.

5 Another method is to potentiate the activity of blood for transfusion, the method comprising adding to the blood a composition comprising one or more thiols.

A different aspect of the invention is a method for identifying a human candidate for a condition associated with a low NO:Hb ratio in red blood cells, said

10 method comprising measuring NO content as S-nitrosothiol and as iron nitrosylhemoglobin in red blood cells isolated from a human, measuring hemoglobin content in the red blood cells isolated from the human, determining a NO:Hb ratio, and comparing the NO:Hb ratio to a normal mean value for NO:Hb, wherein if the NO:Hb ratio is significantly below the normal mean value, the human is a candidate for a

15 condition associated with a low NO:Hb ratio in red blood cells.

In any of the methods for raising NO:Hb or raising NO:Hb as SNO, or for alleviating the effects of a medical intervention or treating a medical condition, wherein a composition comprising NO, a composition comprising an inorganic nitrite, a composition comprising an organic nitrite, or a composition comprising a donor of

20 biologically active NO is administered to the mammal, the method can comprise additional steps to monitor the progress of the effects of the treatment. For example, blood of the mammal can be isolated and analyzed for hemoglobin and for NO in SNO and NO in iron nitrosylhemoglobin to determine whether a desired value or ratio or improvement has been achieved, e.g., whether a desired value of NO:Hb as SNO has

25 been reached. Further steps can be taken to give additional doses of the pharmaceutical composition, if necessary, and the analysis of the blood can be repeated, if necessary.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D are spectrographs of different forms of Hb as described in Example 1.

Figure 2A is a graph showing formation, with time, of SNO-Hb by S-nitrosylation.

Figure 2B is a graph showing the decomposition, with time, of oxy and deoxy forms of SNO-Hb.

Figure 3A is a graph showing the loading of red blood cells with S-nitrosocysteine, over time. The inset is a series of spectrographs of forms of Hb as described in Example 3.

Figure 3B is a series of tracings recording isometric tone of a rabbit aortic ring following treatment of the aortic ring with various agents as described in Example 3.

Figure 4A is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the experiment.

Figure 4B is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the experiment, wherein glutathione was also added to test the effect as compared to Figure 4A.

Figure 4C is a graph of the ratio of S-nitrosoglutathione formed/starting SNO-Hb concentration versus time, showing rates of NO group transfer from oxy and met forms of Hb to glutathione.

Figure 4D is a graph of S-nitrosothiols exported from loaded red blood cells over time.

Figure 5 is a graph showing the mean arterial blood pressure in rats after they received various doses of oxyHb (▲), SNO-oxyHb (■), or SNO-metHb (●).

Figures 6A-6F are a series of tracings recording blood pressure (Figures 6A and 6B), coronary artery diameter (Figures 6C and 6D) and coronary artery flow (Figures 6E and 6F), after administration of S-nitrosohemoglobin to anesthetized dogs.

Figures 7A and 7B are graphs showing the contractile effects of oxyHb, SNO-oxyHb, deoxy-Hb and SNO-deoxy-Hb on thoracic aortic ring isolated from rabbit. Measurements are shown as percent increase in tension of aortic ring as a function of the log of the concentration of hemoglobin or SNO-hemoglobin. Measurements were 5 made after the tension had stabilized.

Figure 7C is a graph showing the percent change in tension of contracted aortic ring as a function of the log concentration of SNO-hemoglobin at the concentrations of O<sub>2</sub> indicated, in addition to 10 μM glutathione.

Figure 7D is a graph showing the percent change in tension of contracted aortic 10 ring as a function of the log concentration of SNO-glutathione, in the concentrations of O<sub>2</sub> indicated.

Figure 8A and Figure 8B are each a series of four graphs illustrating the change 15 with time in tension of rabbit aortic ring upon the addition of red blood cells treated with S-nitrosocysteine ("red blood cells loaded with nitric oxide"), or untreated red blood cells, as indicated, in the concentration of O<sub>2</sub> indicated.

Figure 8C is a graph illustrating the change with time in tension of rabbit aortic ring contracted with phenylephrine under hypoxic conditions (6-7 torr) and then exposed to either 1 μM Hb or SNO-Hb.

Figure 9 is a bar graph depicting the concentrations of FeNO/Hb and SNO/Hb in 20 venous or arterial blood as measured in Example 11. ATA = atmospheres of absolute pressure.

Figures 10A-10I are each graphs showing the effects of SNO-Hb (●) and Hb (■) (1 μmol/kg infused over 3 minutes) on local blood flow in substantia nigra (SN), caudate putamen nucleus, and parietal cortex of rats, in 21% O<sub>2</sub> (Figures 10A, 10B and 25 10C), in 100% O<sub>2</sub> (Figures 10D, 10E and 10F), and in 100% O<sub>2</sub> at 3 atmospheres absolute pressure (Figures 10G, 10H and 10I) as measured in Example 12.

Figure 11A is a bar graph showing the percent change in blood pressure of rats, during exposure to three different conditions (inspired O<sub>2</sub> concentrations of 21%, 100%,

or 100% O<sub>2</sub> at 3 ATA) upon infusion of GSNO, SNO-Hb, or Hb, as tested in Example 13.

Figure 11B is a bar graph showing the percent change in blood pressure of rats [pre-administered (+ L-NMMA), or not preadministered (- L-NMMA), N<sup>G</sup>-monomethyl-L-arginine] upon infusion of SNO-RBCs, as tested in Example 13.

Figures 12A, 12B and 12C are graphs showing the ratio of NO content in the blood to hemoglobin in blood samples, in the form of SNO-hemoglobin (Figure 12A), iron-nitrosylhemoglobin (Figure 12B) or total NO bound to hemoglobin (Figure 12C). Normal: samples drawn from different normal human subjects. PPH: samples drawn from different human subjects with primary or secondary pulmonary hypertension. Also shown in each graph are the mean values.

Figure 13 is a bar graph in which the proportion of total biologically active NO in red blood cells in the form of S-nitrosothiol is shown for red blood cells stored at room temperature for the time shown.

Figure 14 is a bar graph showing the ratio of NO content in the blood to hemoglobin in blood samples, in fresh blood and in blood that has been frozen for 4 days. Also shown within each bar is biologically active NO measured as arterial S-nitrosothiol (white portion) and biologically active NO measured as arterial iron nitrosylhemoglobin (black portion).

Figure 15 is a bar graph showing the molar ratio of NO content to hemoglobin in blood samples, in blood drawn from a patient with PPH and in blood drawn from the patient following exposure to exposure to 0.125% ethyl nitrite inhaled for 10 minutes. Also shown within each bar are biologically active NO measured as S-nitrosothiol (white portion) and biologically active NO measured as iron nitrosylhemoglobin (shaded portion).

Figure 16 shows plots of blood vessel tone of a rabbit aortic ring system in which the aortic ring was perfused with (left, "patient") blood from a patient with PPH, and (right, "NO") blood from the patient following treatment of the patient with ethyl

nitrite as in Figure 15, under conditions of 1% oxygen. The downward deflection on the plot indicates a dilation of the blood vessel, which for the "NO" blood, occurred for approximately 30 seconds.

Figure 17 is a bar graph showing the change in coronary flow, left ventricular  
5 end diastolic pressure (LVEDP) and blood pressure in a dog, following an infusion of  
red blood cells treated with NO. See Example 18.

Figure 18 is a bar graph showing the change, as percent, in pulmonary artery  
(PA) pressure measured in isolated, buffer-perfused rabbit lung, in hypoxia (12% O<sub>2</sub>),  
and upon the addition to the perfusate of untreated red blood cells (RBC), red blood  
10 cells treated with S-nitrosocysteine (SNO-RBC) or iron nitrosylhemoglobin (NO-RBC)  
as in Example 19.

Figure 19 is a bar graph showing the percent relaxation in aortic rings, as  
described in Pawloski, J.R. *et al.*, *Nature* 409:622-625 (2001). Blood was taken from  
human subjects with varying severities of sickle cell disease and normal human control  
15 subjects. The same blood was also studied after exposure to NO (1:250 NO:Hb).

Figures 20A and 20B are recordings of coronary blood flow in dogs, measured  
by pulse Doppler flow probe, under 95% O<sub>2</sub> (normoxia; Figure 20A) or ischemia (60%  
O<sub>2</sub>; Figure 20B). The arrows show the time of infusion of red blood cells treated with  
NO as in Example 21 (NO-RBC), or untreated red blood cells (RBC).

20 Figure 21 is a bar graph representing the change in coronary blood flow in a dog,  
in response to an infusion into the circumflex coronary artery of untreated (native) red  
blood cells or NO-loaded red blood cells, under normoxia (unshaded bar) or hypoxia  
(shaded bar). Direct measurements shown in Figures 20A and 20B have been replotted.  
See Example 21.

25 Figure 22 is a bar graph showing the results of the experiments in which S-  
nitrosocysteine ethyl ester was added to human red blood cells, as described in Example  
22.

DETAILED DESCRIPTION OF THE INVENTION

Roles for Hemoglobin in Physiology

The increase in SNO-Hb content of red cells across the pulmonary circuit (right ventricular import-left ventricle) suggests that the Hb molecule is S-nitrosylated in the lung. Selective transfer of the NO group from endogenous RSNOs found in lung (Gaston, *et al.* (1993)) and blood (Scharfstein, J.S. *et al.*, *J. Clin. Invest.* 94:1432-1439 (1995)) to SH groups of Hb, substantiate these findings. The corresponding decline in Hb(FeII)NO levels across the pulmonary bed reveals a role for the lung either in the elimination of NO or in its intramolecular transfer from heme to cys $\beta$ 93. Taken in aggregate, these data extend the list of function-regulating interactions of Hb with small molecules within the respiratory system, previously known to include the elimination of CO and CO<sub>2</sub>, and uptake of O<sub>2</sub>. Since, as demonstrated herein, oxygenation of Hb leads to structural changes that increase the NO-related reactivity of cys $\beta$ 93, O<sub>2</sub> can now be regarded as an allosteric effector of Hb S-nitrosylation.

15        The arterial-venous difference in SNO-Hb concentration suggests that the protein acts as an NO group donor in the systemic circulation. There is good indication that SNO-Hb functions in regulation of vasomotor tone. In the microcirculation, where control of blood pressure is achieved, erythrocytes come in intimate contact with endothelial surfaces. Under these conditions, Hb can contract the vasculature by sharply decreasing the steady state level of free NO (Lancaster, J.R., (1994)). This is believed to contribute to the increases in blood pressure that occur with infusion of cell-free Hbs (Vogel, W.M., *et al.*, *Am. J. Physiol.* 251:H413-H420 (1986); Olsen, S.B., *et al.*, *Circulation* 93:329-332 (1996)). The transient nature of such hypertensive responses, however, is consistent with the subsequent formation of SNO-Hb which counteracts this effect, evidenced by its lowering of blood pressure at naturally occurring concentrations. Thus, the capacity of the erythrocyte to support the synthesis and metabolism of SNO-Hb is important for normal blood flow.

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Mammals must have adopted unique molecular mechanisms to ensure adequate NO delivery in the microcirculation. Results herein suggest that Hb has evolved both electronic and conformational switching mechanisms to achieve NO homeostasis. Specifically, NO scavenging by the metal center(s) of SNO-Hb(FeII) $O_2$  is sensed

5 through its conversion to met(FeIII) (Figure 1B). This electronic switch effects decomposition of SNO-Hb with NO group release (Figures 3A, 3B, 4A). In this manner, the NO-related activity of SNO-Hb is partly determined by the amount of NO scavenged. Changes in  $O_2$  tension also function to regulate NO delivery, as it is observed herein that NO release is facilitated by deoxygenation. This allosteric effect

10 promotes the efficient utilization of  $O_2$ , as NO controls mitochondrial respiration (Shen, W., *et al.*, *Circulation* 92:3505-3512 (1995)).

S-nitrosothiol groups in proteins have been implicated in NO metabolism and in regulation of cellular functions (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci USA* 89:444-448 (1992); Stamler, J.S., *Cell* 78:931-936 (1994)). The identification of SNO-Hb in

15 erythrocytes is the first demonstration of an intracellular S-nitrosoprotein and gives further credence to the role of such proteins in cellular regulation. The question arises as to how SNO-Hb relaxes blood vessels when any free NO released would be scavenged instantaneously by Hb itself, according to previous theories (Lancaster, J.R., (1994)). Noteworthy in this regard are studies showing that RSNO activity involves

20 nitrosyl ( $NO^+$ ) transfer to thiol acceptors (Scharfstein, J.S., *et al.*, (1994); Arnelle, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.* 318:279-285 (1995); Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:7674-7677 (1992)), which serve to protect the NO-related activity from inactivation at metal centers. Findings presented herein indicate that S-nitrosothiol/thiol exchange with glutathione (forming GSNO) occurs within

25 erythrocytes, and is influenced by the oxidation state of heme and its occupation by ligand. Certain activities of GSNO in bacteria require transport of intact dipeptide (i.e., S-nitrosocysteinylglycine) across the cell membrane (DeGroote, M.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995)). The data presented below in the Examples show

that S-nitrosothiol transport occurs also in eukaryotic cells. GSNO, or related thiol carriers exported by erythrocytes (Kondo, T., *et al.*, *Methods in Enzymology*, Packer, L., ed., Academic Press, 252:72-83 (1995)), might also initiate signalling in or at the plasmalemma (Stamler, J.S., *Cell* 78:931-936 (1994)), given reports of thiol-dependent activation of potassium channels by EDRF (Bolotina, V.M., *et al.*, *Nature* 368:850-853 (1994)). Alternative possibilities also merit consideration. In particular, reports that Hb associates with red cell membranes via cys $\beta$ 93 (Salhany, J.M. and Gaines, K.C., *Trends in Biochem. Sci.*, pp. 13-15, Jan. (1981)) places Hb in a position to donate the NO group directly to contacting endothelial surfaces, perhaps via SNO/SH exchange. Cell surface interactions appear to be operative in signaling mediated by other S-nitrosoproteins (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992); Stamler, J.S., *Cell* 78:931-936 (1994)).

The highly conserved Cys $\beta$ 93 residues in Hb influence the oxygen affinity and redox potential of the heme iron and its physiochemical properties (Garel, C., *et al.*, *Biochem.* 123:513-519 (1982); Jocelyn, P.C., *et al.*, *Biochemistry of the SH Group*, p. 243, Academic Press, London; (1972); Craescu, C.T., *J. Biol. Chem.* 261:14710-14716 (1986); Mansouri, A., *Biochem. Biophys. Res. Commun.* 89:441-447 (1979)). Nonetheless, their long sought-after physiological function has remained a mystery. The studies herein suggest new sensory and regulatory roles for Hb, in which Cys $\beta$ 93 functions in transducing NO-related signals to the vessel wall. In particular, the physiological function of Cys $\beta$ 93, which is invariant in all mammals and birds, is to deliver under allosteric control, NO-related biological activity that cannot be scavenged by heme. Thus, these data bring to light a dynamic circuit for the NO group in which intraerythrocytic Hb participates as both a sink and a donor, depending on its microenvironment. Such observations provide answers to paradoxes that arise from conceptual frameworks based solely on diffusional spread and reaction of free NO (Lancaster, J.R., (1994); Wood and Garthwaite, *J. Neuropharmacology* 33:1235-1244).

(1994)); and has implications that extend to other thiol- and metal-containing (heme) proteins, such as nitric oxide synthase and guanylate cyclase.

The discoveries reported here have direct therapeutic implications. Specifically, concerns over loss of NO-related activity due to inactivation by blood Hb (Lancaster, 5 J.R., (1994)) are obviated by the presence of an RSNO subject to allosteric control.

SNO-Hb is free of the adverse hypertensive properties of cell-free Hb preparations that result from NO scavenging at the metal centers. A composition comprising one or more of the various forms of cell-free SNO-Hb (e.g., SNO-Hb[FeII]O<sub>2</sub>, SNO-Hb[FeIII], SNO-Hb[FeII]CO) can be administered in a pharmaceutically acceptable vehicle to a human 10 or other mammal to act as a blood substitute.

#### Role of S-Nitrosohemoglobin in Blood Flow Regulation

In the classical allosteric model, Hb exists in two alternative structures, named R (for relaxed, high O<sub>2</sub> affinity) and T (for tense, low O<sub>2</sub> affinity). The rapid transit time of blood through the capillaries requires that Hb assume the T-structure to efficiently 15 deliver O<sub>2</sub> (M.F. Perutz, pp. 127-178 in *Molecular Basis of Blood Diseases*, G. Stamatayianopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G., pp. 215-235 (John Wiley & Sons Inc., New York, 1995). The switch from R to T in RBCs normally takes place when the second molecule of O<sub>2</sub> is liberated. This allosteric transition also controls the reactivity of two highly conserved cysteine $\beta$ 93 20 residues that can react with 'NO'. Thiol affinity for NO is high in the R-structure and low in T. This means that the NO group is released from thiols of Hb in low PO<sub>2</sub> and explains the arterial-venous (A-V) difference in the S-nitrosohemoglobin (SNO-Hb) level of blood (see Table 2, Example 8). A major function of (S)NO in the vasculature is to regulate blood flow, which is controlled by the resistance arterioles (Guyton, A.C., 25 in *Textbook of Medical Physiology* (W.B. Saunders Co., Philadelphia, 1981) pp. 504-513). It is shown from the Examples herein that (partial) deoxygenation of SNO-Hb in these vessels (Duling, B. and Berne, R.M. *Circulation Research* 27:669 (1970); Popel,

A.S., et al., (erratum *Am. J. Physiol.* 26(3) pt. 2). *Am. J. Physiol.* 256:H921 (1989); Swain, D.P. and Pittman, R.N. *Am. J. Physiol.* 256:H247-H255 (1989); Torres, I. et al., *Microvasc. Res.* 51:202-212 (1996); Buerk, D. et al., *Microvasc. Res.* 45:134-148 (1993)) actually promotes O<sub>2</sub> delivery by liberating (S)NO. That is, the allosteric transition in Hb functions to release (S)NO in order to increase blood flow.

O<sub>2</sub> delivery to tissues is a function of the O<sub>2</sub> content of blood and blood flow (Dewhirst, M.W. *et al.*, *Cancer Res.* 54:3333-3336 (1994); Kerger, H. *et al.*, *Am. J. Physiol.* 268:H802-H810 (1995)). Blood oxygen content is largely determined by Hb, which undergoes allosteric transitions in the lung and systemic microvasculature that 10 promote the binding and release of O<sub>2</sub> (L. Stryer, in *Biochemistry*, L. Stryer, Ed. (W.H. Freeman & Co., San Francisco, 1981) pp. 43-82; Guyton, A.C. in *Textbook of Medical Physiology* (W.B. Saunders Co., Philadelphia, 1981); Perutz, M.F., pp. 127-178 in *Molecular Basis of Blood Diseases*, G. Stamatyanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G. (John Wiley & Sons Inc., New York, 1995) 15 pp. 215-235, pp. 208-215, 224-225, 230-245, 344-355)). Intimate contact between erythrocyte and endothelium is believed to facilitate O<sub>2</sub> delivery by minimizing the distance for O<sub>2</sub> diffusion into surrounding tissues (Caro, C.G. *et al.*, Oxford University Press, Oxford, 363 (1978)). On the other hand, regional blood flow is regulated by metabolic requirements of the tissue: blood flow is increased by hypoxia and decreased 20 when O<sub>2</sub> supply exceeds demand (Guyton, A.C., in *Textbook of Medical Physiology* (W.B. Saunders Co., Philadelphia, 1981) pp. 504-513)). These classical physiological responses are thought to be partly mediated by changes in the level of endothelial-derived NO and its biological equivalents (Park, K.H. *et al.*, *Circ. Res.* 71:992-1001 25 (1992); Hampl, V. *et al.*, *J. Appl. Physiol.* 75(4):1748-1757 (1993)).

This standard picture is incomplete. First, it has been puzzling that significant O<sub>2</sub> exchange occurs in the precapillary resistance vessels (evidenced by the periarteriolar O<sub>2</sub> gradient; Duling, B. and Berne, R.M. *Circulation Research* 27:669 (1970); Popel, A.S., *et al.*, (erratum *Am. J. Physiol.* 26(3) pt. 2). *Am. J. Physiol.* 256, H921 (1989);

Swain, D.P. and Pittman, R.N. *Am. J. Physiol.* 256:H247-H255 (1989); Torres, I. *et al.*, *Microvasc. Res.*, 51:202-212 (1996); Buerk, D. *et al.*, *Microvasc. Res.*, 45:134-148 (1993)). Why is O<sub>2</sub> lost to counter-current venous exchange prior to reaching the tissues? Second, close contact between endothelial surfaces and erythrocytes leads to 5 sequestration of NO by Hb (Stamler, J.S., *Nature* 380:108-111 (1996); Perutz, M.F., *Nature* 380:205-206 (1996)). Decreases in the steady-state levels of NO in terminal arterioles (King, C.E. *et al.*, *J. Appl. Physiol.* 76(3):1166-1171 (1994); Shen, W. *et al.*, *Circulation* 92:3505-3512 (1995); Kobzik, L. *et al.*, *Biochem. Biophys. Res. Comm.* 211(2):375-381 (1995); Persson, M.G., *et al.*, *Br. J. Pharmacol.* 100:463-466 (1990) 10 and capillaries (Mitchell, D., and Tyml, K., *Am. J. Physiol. 270 Heart Circ. Physiol.* 39:H1696-H1703 (1996)) contract blood vessels, blunt hypoxic vasodilation and reduce red cell velocity. This line of reasoning leads to the paradox: the red blood cell seems to oppose its own O<sub>2</sub> delivery function (note *in vivo* effects of Hb in Figures 10A-10I).

The finding that the O<sub>2</sub> gradient in precapillary resistance vessels promotes NO 15 group release from SNO-Hb appears to answer these questions. SNO-Hb compensates for NO scavenging at the heme iron by assuming the T-structure which liberates SNO. Specifically, Cys93 donates the NO group in deoxy structure whereas it cannot do so in the oxy conformation. Accordingly, the O<sub>2</sub> gradient determines whether SNO-Hb dilates or constricts blood vessels. Stated another way, SNO-Hb senses the tissue PO<sub>2</sub> 20 (i.e., the periarteriolar O<sub>2</sub> gradient) and then utilizes the allosteric transition as a means to control arteriolar tone. If the tissue is hypoxic (i.e., the O<sub>2</sub> gradient is high), SNO is released to improve blood flow. However, if O<sub>2</sub> supply exceeds demand (i.e., the O<sub>2</sub> gradient is small), SNO-Hb holds on to the NO group by maintaining the R-structure--with the net effect of reducing blood flow in line with demand. SNO-Hb thereby 25 contributes to the classical physiological responses of hypoxic vasodilation and hyperoxic vasoconstriction.

Based on studies described herein, especially Examples 9-13, the following picture emerges. Partially nitrosylated Hb (Hb[FeII]NO) enters the lung in T-structure

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(see venous measurements in Figure 9). There, S-nitrosylation is facilitated by the O<sub>2</sub>-induced conformational change in Hb. SNO-oxyHb (SNO-Hb[FeII]O<sub>2</sub>) enters the systemic circulation in R-structure (see arterial levels in Figure 9). Oxygen losses in precapillary resistance vessels then effect an allosteric transition (from R to T) in Hb which liberates 'NO' to dilate blood vessels (see especially Figures 7D and 10A-C). NO released from Hb can be transferred directly to the endothelium, or by way of low mass S-nitrosothiols--such as GSNO--which are exported from RBCs (see especially Figure 4D and Example 4; see also Figures 7C and 11A). Thus, the O<sub>2</sub> gradient in arterioles serves to enhance O<sub>2</sub> delivery: it promotes an allosteric transition in Hb which releases NO-related activity to improve blood flow.

#### Further embodiments

The subject invention relates to a method of loading cells with a nitrosating agent as exemplified for red blood cells as in Figure 3A, but which can be accomplished in more ways. Suitable conditions for pH and for the temperature of incubation are, for example, a range of pH 7-9, with pH 8 being preferred, and a temperature range of 25 to 37°C. For red blood cells, short incubation times of 1 to 3 minutes are preferred if it is desired to limit the formation of S-nitrosated forms of Hb. However, intracellular concentrations of 1 mM nitrosating agent can be reached.

The nitrosating agent should be a good donor of NO<sup>+</sup> and should be able to diffuse through the cell membrane of the target cell type. That is, it is preferably of low molecular weight, in comparison to the molecular weight of S-nitrosoproteins. Examples are S-nitroso-N-acetylcysteine, S-nitrosocysteinylglycine, S-nitrosocysteine, S-nitrosohomocysteine, organic nitrates and nitrites, metal nitrosyl complexes and other related nitrosating agents as defined in "Donors of Nitrogen Oxides" pp. 71-119 In *Methods in Nitric Oxide Research* (Feelisch, M. and Stamler, J.S., eds.) Wiley, Chichester, U.K. (1996), the contents of which chapter are hereby incorporated by reference in their entirety. Nitrosating agents have differential activities for different

reactive groups on metal-containing proteins. A nitrosating agent can be chosen for minimal oxidation of the heme iron of Hb, and maximum activity in nitosylating thiol groups such as found on cysteine. Assay methods are available for detection of nitrosation products, including S-nitrosothiols. See Stamler, *et al.*, U.S. Patent Number 5,459,076, the contents of which are hereby incorporated by reference in their entirety. See also, for example Keefer, L.K., and Williams, D. L. H., "Detection of Nitric Oxide Via its Derived Nitrosation Products," chapter 35, pp. 509-519 *In Methods in Nitric Oxide Research* (Feelisch, M. and Stamler, J.S., eds.) John Wiley and Sons, Ltd., Chichester, U.K., 1996; see also Stamler, J.S. and Feelisch, M., "Preparation and 10 Detection of S-Nitrosothiols," chapter 36, pp. 521-539, *ibid.* Nitrite and nitrate products can be assayed by methods described, for instance, in Schmidt, H.H.H.W. and Kelm, M., "Determination of Nitrite and Nitrate by the Griess Reaction," chapter 33, pp. 491-497, *ibid.*, and in Leone, A.M. and Kelm, M., "Capillary Electrophoretic and Liquid Chromatographic Analysis of Nitrite and Nitrate," chapter 34, pp. 499-507, *ibid.*

15 Such low molecular weight nitrosating agents can be used in red blood cells to deliver NO-related activity to tissues in a mammal. Treatment of red blood cells with nitrosating agent further serves to increase the O<sub>2</sub> delivery capacity of red blood cells. Such treatment of red blood cells also allows for the scavenging of oxygen free radicals throughout the circulation. It is possible to load red blood cells with S-nitrosothiol, for 20 example, by a process of removing whole blood from a patient's body (as a minimal method of isolating the red blood cells) treating the red blood cells with low molecular weight nitrosating agent, such as by incubating the red blood cells in a solution of low molecular weight nitrosating agent, and then reintroducing the red blood cells into the same patient, thereby allowing the treatment of a number of types of diseases and 25 medical disorders, such as those which are characterized by abnormal O<sub>2</sub> metabolism of tissues, oxygen-related toxicity, abnormal vascular tone, abnormal red blood cell adhesion, and/or abnormal O<sub>2</sub> delivery by red blood cells. Such diseases can include, but are not limited, to ischemic injury, hypertension, shock, angina, stroke, reperfusion

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injury, acute lung injury, sickle cell anemia, schistosomiasis and malaria. The use of such "loaded" red blood cells also extends to blood substitute therapy and the preservation of living organs, such as organs for transplantation, for example. In some cases, it can be appropriate to treat a patient with loaded red blood cells originating from  
5 a different person.

A particular illustration of the mechanism of the treatment method is presented here by considering sickle cell anemia. Sickle cell patients suffer from frequent vascular occlusive crises which manifest in clinical syndromes such as the acute chest syndrome and hepatic dysfunction. Both endothelial cell dysfunction, resulting in a  
10 clotting diathesis as well as dysfunction intrinsic to the red blood cell, are central to disease pathogenesis. At the molecular level, the increased expression of vascular adhesion molecules such as VCAM promote the adhesion of sickled red blood cells containing abnormal hemoglobin. It follows that decreasing cytokine expression on endothelial cells, promoting endothelial function and attenuating red cell sickling, are  
15 key therapeutic objectives. However, currently used therapies have been generally unsuccessful.

In this novel method for loading red blood cells with intracellular NO-donor S-nitrosothiols, the effect is to increase oxygen affinity -- which in and of itself should attenuate red blood cell sickling -- and to endow the red blood cell with vasodilator and  
20 antiplatelet activity, which should reverse the vasoocclusive crisis. Moreover, nitric oxide should attenuate the expression of adhesion molecules on endothelial cell surfaces, thus restoring endothelial function.

Herein is described a novel therapeutic approach to the treatment of sickle cell disease which involves loading of red blood cells with S-nitrosothiols or other  
25 nitrosating agents. Two examples of therapeutic approaches are given. In the first, the patient's own red blood cells are S-nitrosylated extracorporeally (yielding "loaded" red blood cells) and then given to the patient. The second approach is to directly administer to a patient an agent such as S-nitrosocysteine, which is permeable to red blood cells.

For some diseases or disorders, the administration of NO-loaded red blood cells is especially desirable. Upon a change from the oxygenated to the deoxygenated state, or upon a change in the oxidation state of the heme Fe from the reduced state (FeII) to the oxidized (FeIII) state, NO is released from the thiol groups of hemoglobin, and is 5 rapidly transferred to glutathione to form S-nitrosoglutathione. Red blood cells are known to have a high concentration of glutathione. S-nitrosoglutathione efficiently delivers NO in a biologically active form to tissues.

In another aspect, the invention is a method for the treatment of infection by administering to an infected mammal an agent which causes S-nitrosylation of thiol 10 groups within the cells which are the target of such agent. For example, an S-nitrosothiol to which lymphocytes are highly permeable can be administered to a patient infected with HIV. Such treatment for HIV can also be used extracorporeally, to blood isolated from the patient. In another application, the infection is bacterial, and the S-nitrosothiol to be used as an anti-bacterial agent is one to which the target bacterial cells 15 are highly permeable, as compared to the permeability properties of the host cells. (see, for example, DeGroote, M.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995).) Alternatively, nitrosothiols can be used to treat *Plasmodium falciparum* within red blood cells.

Another embodiment of the invention is a method for specifically modifying a 20 protein containing one or more metal atoms so that the protein becomes S-nitrosylated at one or more thiol groups without modifying the metal, as by changing the oxidation state or causing the metal atoms to bind NO. This can be accomplished by the use of a reagent which possesses NO<sup>+</sup> character, such as a nitrosothiol (see, for instance, Example 4A), which reacts specifically with thiol groups of a protein in which metal is 25 bound.

For hemoglobin, the nitrosation method does not affect the heme. SNO-oxyHb (SNO-Hb(FeII)O<sub>2</sub>) can be synthesized from Hb(FeII)O<sub>2</sub> with up to 2 SNO groups per tetramer without oxidation of the heme Fe from FeII to FeIII. In contrast, when

Hb(FeII)O<sub>2</sub> is incubated with excess nitric oxide or nitrite, methemoglobin (HbFe[III]) forms rapidly (Example 1B) and to a significant extent. When Hb[FeII] is incubated with nitric oxide, NO binds rapidly to the heme, forming Hb(FeII)NO to a significant extent (Example 1A).

5        Although rates of formation of SNO-Hb(FeII)O<sub>2</sub> from Hb(FeII)O<sub>2</sub> are more rapid (see Example 2A), the corresponding SNO-deoxyHb form can also be made by incubation of S-nitrosoglutathione or S-nitrosocysteine, for example, with Hb(FeII), yielding SNO-Hb(FeII), as in Example 1C.

10      The effects of the various forms of Hb on vasodilation -- constriction, dilation or a neutral effect -- depend on three factors: whether 1) the Fe of the heme is oxidized, 2) O<sub>2</sub> is bound at the heme (that is, the oxygenation state, dictated by the conformation of the protein as R state or T state), and 3) thiol is present in sufficient concentration to facilitate the transfer of NO<sup>+</sup>.

15      The importance of the first factor is shown in Figure 4A. Hb(FeII)O<sub>2</sub> and SNO-Hb[FeII]O<sub>2</sub> act as vasoconstrictors, but SNO-Hb[FeIII] (met form, where FeII has been oxidized to FeIII) acts as a vasodilator. Figure 4A shows that SNO-Hb[FeII]O<sub>2</sub>, with oxygen bound at the heme, and with a ratio of SNO/Hb=2, acts as a powerful vasoconstrictor.

20      SNO-Hb(FeII) is also a vasodilator. Figure 2B illustrates the second factor in demonstrating that rates of RSNO decomposition and transfer are much faster for SNO-Hb in the deoxy state than for SNO-Hb in the oxy state.

25      It can be seen how the NO<sup>+</sup>-donating properties of SNO-Hb depend on oxygen concentrations. SNO-Hb releases oxygen at sites of low oxygen concentration or under oxidizing conditions. SNO-Hb releases its NO group(s) to cause vasodilation either due to 1) oxidation of the heme Fe to FeIII or 2) loss of the O<sub>2</sub> from the heme by deoxygenation. It is shown in Figure 2B that NO is transferred off SNO-Hb best in the deoxy state. In ischemia, SNO-Hb deoxygenates, rapidly followed by the loss of NO. It can be seen from the data that SNO-metHb having a ratio of 1 SNO/SNO-metHb is a

more powerful vasodilator than SNO-oxyHb having a ratio of 2 SNO/SNO-oxyHb. It should be noted that S-nitrosylation of Hb induces the R state (oxy conformation). Thus, it follows that 1 SNO-oxyHb molecule having a ratio of 1 SNO/SNO-oxyHb is less potent than 10 molecules of SNO-oxyHb having a ratio of 0.1 SNO/SNO-oxyHb.

5       The third factor is illustrated by the results shown in Figure 4B. These results demonstrate potentiation by thiol of the vasodilator effect of SNO-Hb(FeII)O<sub>2</sub> and SNO-Hb(FeIII). Transfer of NO<sup>+</sup> from SNO-Hb to low molecular weight nitrosothiols is more efficient when Hb is in the deoxy state compared to the oxy state (Figure 2B) or in the met state compared to the oxy state (Figure 4C).

10      It is thought that NO is released or transferred as NO<sup>+</sup> (nitrosyl cation) from SNO-Hb. The SNO groups of SNO-Hb have NO<sup>+</sup> character. Transfer of NO<sup>+</sup> from SNO-Hb occurs most efficiently to small thiols, such as glutathione, and is most efficient when the heme is oxidized (SNO-metHb) or the SNO-Hb is in the deoxy state.

15      One embodiment of the invention resulting from these findings is a method of therapy that enhances the transfer of NO<sup>+</sup> from SNO-Hb to low molecular weight thiols, thereby delivering NO biological activity to tissues, by the coadministration of low molecular weight thiols, along with a form of SNO-Hb, to a mammal in need of the physiological effects of NO. To further increase the effect of NO release it is preferred that the SNO- forms of metHb or deoxyHb (or an equivalent conformation or spin state) 20 be administered with the thiol (See Figure 2B, for example.) A mixture of SNO-metHb and SNO-oxyHb, and possibly also thiol, can also be used. The composition and proportion of these components depends on the disease state. For example, to achieve both enhanced O<sub>2</sub> delivery and NO delivery, SNO-oxyHb can be used. Where no further delivery of O<sub>2</sub> is desirable, as in acute respiratory distress syndrome, for 25 example, the SNO- forms of metHb and deoxyHb are especially preferred.

      Alternatively, the ratios of SNO/Hb can be regulated to control O<sub>2</sub> release.

      The vessel ring bioassay data of Figure 4A agree well with the *in vivo* data of Figure 5. The results of the experiments described in Example 5 confirm that

Hb(FeII)O<sub>2</sub> (oxyHb) causes an increase in blood pressure *in vivo*, as it did also *in vitro*. SNO-Hb(FeIII) (SNO-metHb) causes a decrease in blood pressure *in vivo* as well as *in vitro*. SNO-Hb(FeII)O<sub>2</sub> (SNO-oxyHb) has a negligible effect on blood pressure *in vivo* in contrast to the increase in tension seen in the corresponding vessel ring bioassay. For 5 SNO-oxyHb the *in vivo* effect is neutral. This can be explained by the constrictive effect caused by NO becoming bound to the heme being compensated by the release of NO upon deoxygenation. Therefore, SNO-oxyHb can deliver O<sub>2</sub> with minimal effect on blood pressure.

With knowledge of the results herein, it is possible to synthesize Hb proteins 10 with predicted NO-releasing properties which will constrict, dilate, or have no effect on blood vessels. An additional option is the choice between making oxygenated or deoxygenated forms to administer for medical conditions in which O<sub>2</sub> delivery is desirable, or undesirable, respectively.

It is possible to produce a variety of modified Hbs having specific desired 15 properties of O<sub>2</sub> and NO delivery. For example, Hb in the R state (oxyHb) can be converted to the T state (deoxyHb) by a number of known methods. This can be done, for example, by reaction of Hb with inositol hexaphosphate. It is also known to those skilled in the art that Hb in the R state can be made, for example, by treating Hb with carboxypeptidase. Similarly, it is known that metHb can be synthesized using 20 ferricyanide or nitrite.

Producing Hb molecules which are locked in the T state allows the synthesis of RSNO-Hb which remains in a form that is a biologically active donor of NO, rather than a carrier of NO. Hb which is locked in the R state can be used as a substrate for the synthesis of RSNO-Hb which carries a maximum amount of NO per molecule.

25 Another embodiment of the invention is a blood substitute comprising one or more forms of Hb which have been specifically S-nitrosylated to some extent at one or more thiol groups of the Hb, in order to regulate O<sub>2</sub> release and NO release. Conditions to be treated include those in which NO or O<sub>2</sub> delivery is desired, those in which NO or

O<sub>2</sub> utilization is desired, or those in which NO or O<sub>2</sub> is in excess. For example, in a medical condition which is characterized by the presence of an excess of oxygen free radicals and excess NO·, both the heme of SNO-Hb and NO released by SNO-Hb serve to trap oxygen free radicals. The heme Fe is oxidized in the process of scavenging

5      oxygen free radicals and NO·, and NO is released from the oxidized Hb by donation to a thiol, in the form of RSNO<sup>+</sup> (nontoxic). Inflammation and reperfusion injury, for example, are characterized by excess NO production and an excess of oxygen free radicals. Forms of Hb scavenge oxygen radicals and free NO, converting NO to forms that are not toxic.

10     A further embodiment of the invention is a method of therapy for a condition that would benefit from the delivery of NO biological activity or O<sub>2</sub> or both, based on the administration of a blood substitute comprising a form of SNO-Hb. For example, SNO-Hb is useful to treat myocardial infarction. SNO-Hb has the effect of donating NO, keeping blood vessels open. SNO-Hb deoxygenates at low oxygen tension,

15     delivering oxygen and releasing NO at the same site, thereby causing vasodilation. (See Example 7 and Figures 6A-6F.) These effects can be augmented by also administering thiol, either simultaneously with SNO-Hb, or before or after. For the purpose of treating myocardial infarction, for example, a high concentration or dose of SNO-Hb that has a low ratio of SNO/SNO-Hb is appropriate. Alternatively, SNO-metHb can be used for

20     this purpose.

In another aspect, the invention is a method of enhancing NO-donor therapy by coadministering SNO-Hb together with a nitroso-vasodilator (nitroglycerin, for example) which would be otherwise consumed by the conversion of oxyHb to metHb in Hb which has not been S-nitrosylated.

25     Interactions between NO and hemoglobin (Hb) have been interpreted in terms of an allosteric mechanism in which NO binding to hemes of Hb is favored in the deoxygenated (T) structure that sequesters NO, whereas NO binding to cysteine thiols occurs in the oxy (R) state, preserving NO bioactivity. Hemoglobin transforms NO that

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it has sequestered from the endothelium, into bioactive S-nitrosothiols (SNO), which are then exported from RBC membranes to dilate blood vessels. RBCs thus optimize O<sub>2</sub> delivery. In normal subjects, Hb[Fe]NO predominates in venous blood, while SNO-Hb levels are higher in arterial blood. The Hb[Fe]NO/SNO-Hb equilibrium depends on Hb  
5 O<sub>2</sub> saturation *in vivo*.

Biologically active NO can be measured as S-nitrosothiol (in the form of SNO-Hb) and iron nitrosylhemoglobin. Red blood cells of normal humans have been found to carry a range of biologically active NO. See Figures 12A, 12B and 12C. According to these measurements on red blood cells of freshly drawn human blood, the mean value  
10 for total NO:Hb as a molar ratio, for NO in the form of S-nitrosothiol and iron nitrosylhemoglobin, is approximately 0.0030. The range of NO:Hb values measured in normal humans was approximately 0.0002 to 0.0060. Desirable ranges of NO:Hb in blood can be, for example, approximately 0.0002 to 0.0010, 0.0010 to 0.0020, 0.0020 to 0.0030, 0.0030 to 0.0040, 0.0040 to 0.0050, and 0.0050 to 0.0060. Desirable values for  
15 NO:Hb in blood can be, for example, approximately 0.0010, 0.0020, 0.0030, 0.0040, 0.0050, or 0.0060, and the mean of approximately 0.0030. A desirable ratio of SNO to total NO is approximately 0.5, as measured in arterial blood. In some cases, it may be desirable or necessary to increase NO:Hb to as much as about 0.04, to bring about a pharmacological or therapeutic effect.

20 A low NO:hemoglobin ratio in red blood cells can be approximately zero, or in a low range of approximately 0.0002 to 0.0010; in other cases, a low NO:hemoglobin ratio can be less than the mean value of approximately 0.0030. Generally, any value lower than two standard deviations below the mean is considered to be below normal. A low NO:Hb ratio will be defined differently, depending on the particular disease state.  
25 In some cases, patients with NO:Hb measured between 0.0002 and 0.0060, otherwise considered to be within a normal range, should be considered for therapy to increase NO:Hb, if there are other indicators of a diagnosis of a condition reported herein to be associated with low NO:Hb.

Normal NO:Hb specifically as SNO in red blood cells is in the range of approximately 0 - 0.007, with the mean approximately 0.0015. See Figure 12A. Desirable ranges of NO:Hb as SNO can be, for example, 0-0.001, 0.001-0.002, 0.002-0.003, 0.003-0.004, 0.004-0.005 and 0.005-0.006. Desirable values for NO:Hb as SNO

5 can be, for example, approximately 0.001, 0.002, 0.003, 0.004, 0.005, or 0.006. In methods of therapy to raise total NO:Hb and to raise NO:Hb as SNO, it is preferable to raise both values to at least the mean value, or to raise SNO levels within the RBCs by at least 2-fold. By an alternative criterion, SNO:iron nitrosyl hemoglobin should reach at least 25% in a method of therapy.

10 In primary pulmonary hypertension, as in other medical conditions characterized by a deficiency in NO bioactivity, the NO:Hb ratio in the red blood cells is low and SNO is deficient compared to the mean normal value ( $p < 0.05$ ). Methods of raising NO:Hb in a mammal, especially a human, can be used to treat a number of conditions and disorders, including, but not limited to, systemic hypertension, pulmonary

15 hypertension, stroke, myocardial infarction, sickle cell disease of humans, sepsis, thalassemias, polycythemia, congenital disorders of red blood cells, coronary disease, and hypoxic conditions (e.g., apnea, V/Q mismatch or low  $pO_2$ ). "Pulmonary hypertension" as used herein refers to not only primary pulmonary hypertension, but also secondary hypertension that occurs, for instance, with Raynaud's phenomenon, in

20 pregnancy, in newborns, in chronic obstructive pulmonary disease, in congenital heart disease, in bronchitis and emphysema, in systemic sclerosis, in mixed connective tissue disease, and in schistosomiasis.

Certain medical interventions, such as treatment with erythropoietin, treatment with blood transfusions using blood in which NO available as S-nitrosothiol has been

25 depleted from red blood cells with time, and treatment with hemoglobin-based blood substitutes in which NO:hemoglobin is low, can also decrease the NO:hemoglobin ratio within red blood cells, leading to morbidity and mortality. For these medical

interventions, as well as for the medical disorders of NO:Hb ratio referred to above, NO:Hb can be monitored and treated to bring about an increase.

As seen in experiments described herein, pulmonary hypertension is characterized by low NO in the red blood cells and SNO is absent. Patients with

5 primary pulmonary hypertension (PPH) show impairments in erythrocyte deformability, compared to the erythrocytes of normal volunteers. These defects increase blood viscosity and impair O<sub>2</sub> delivery, and have been linked to increases in pulmonary vascular resistance (Persson, S.U. *et al.*, *Angiology* 42(10):836-842, 1991). The normal rheological properties of the red blood cell may be necessary for the proper regulation of  
10 arterial pressure in the lung. Abnormalities in the rheological properties of RBCs have been associated with V/Q mismatching, impaired O<sub>2</sub> utilization and increased pulmonary artery pressure (Machiedo, G.W. *et al.*, *Arch. Surg.* 124(12):1386-1389, 1989). In sickle cell disease, for example, as in Raynaud's disease, an elevated arterial pressure is seen in the lung under hypoxic conditions. Among the consequences of  
15 sickle cell disease, in which red cell deformability is altered, are increased arterial resistance in the systemic vascular system and in the lung in particular, and increased ventilation/perfusion mismatching in the lung, leading to impaired oxygen delivery. It is seen by the failure of the red blood cell membrane to normally release NO in both primary pulmonary hypertension and in sickle cell disease, that the proper function of  
20 the red blood cell membrane is critical for the release of oxygen, and for release of NO (transferred to thiols, e.g., glutathione) for control of resistance in blood vessels.

The invention includes methods to raise the ratio of NO:hemoglobin or NO:heme in red blood cells in a mammal, especially a human. The method can include one or more steps to administer to the mammal a composition comprising NO. Nitric oxide (NO) itself can be administered in a composition comprising NO to be inhaled as a gas, or NO can be administered parenterally, for example, intravenously, in a liquid solution, e.g., a saturated solution, into which NO gas has been dissolved. Inorganic or organic nitrites can also be administered in the method, wherein the organic nitrite can

be ethyl nitrite, for example, dissolved in a carrier gas such as oxygen to form a gas solution, to be inhaled. See Example 17. An organic nitrite can also be dissolved in a liquid solution, e.g., in ethanol. A liquid solution comprising nitrite or ethyl nitrite can be administered IV at 0.05 to 0.5 g as a bolus or as a continuous infusion. Ethyl nitrite  
5 is also safely administered orally.

The method can include one or more steps to administer a donor of biologically active NO, that is, a compound that will generate NO or NO<sup>+</sup>, which can be transferred from thiol group to thiol group (transnitrosation), with the intermediates being S-nitrosothiols, as those formed by reaction with cysteine amino acid residues in proteins  
10 found within cells or in body fluids. Donors of biologically active NO can be, for example, S-nitrosothiols such as S-nitrosocysteine, S-nitrosohomocysteine, S-nitrosocysteinylglycine, S-nitroso-N-acetylpenicillamine, and S-nitrosoglutathione, and esters of these compounds, for example ethyl esters. It is not required that the donor of biologically active NO be a compound that is able to pass through the membrane of the  
15 red blood cell. These compounds can be given by parenteral routes, for example, intravenously. A solution of ethyl nitrite or S-nitrosothiol can be infused at 1-100 nmoles/kg.

A further method to raise NO:Hb is the administration to the human of NO as an inhaled gas or the administration to the human of NO dissolved in a physiologically  
20 compatible carrier (a buffer or saline, for example) to be delivered by a parenteral method. A liquid solution of NO can be prepared by dissolving it at 0.1 to 1.9 mM in anaerobic saline. The solution can then be infused at 0.1-100 nmoles/kg. By increasing NO:Hb, preferably restoring the NO:Hb to a value within the normal range, the level of SNO-Hb within the red blood cells is increased. This allows for the facilitation of  
25 transnitrosation reactions by which biologically active NO is transferred out of the red blood cell to exert the physiological effects that have been attributed to nitric oxide or EDRF. See, for example, Pawloski, J.R. *et al.*, *Nature* 409:16738-16745 (2001); Hess,

D.T. et al., *Nature Cell Biol.* 3:E46-#49 (2001); and Mato, J.M. et al., *Nature Medicine* 7(10):1107-1108 (2001).

In the lung, using O-nitrosoethanol in experimental systems, S-nitrosothiols (SNOs) have been shown to serve in matching ventilation to perfusion (Moya, M.P. et al., *Proc. Natl. Acad. Sci. USA* 98:5792-5797, 2001). SNOs are delivered to the lung and throughout the vascular system by red blood cells. Therefore, methods to raise NO:Hb in red blood cells or SNO:Hb in red blood cells will allow the normal delivery of SNOs to the lung, lowering pulmonary vascular resistance. Methods to increase NO:Hb will not cause the toxic effects of reactive oxygen species that can occur with inhaled NO therapy, which can include inflammation, hemorrhage, and edema.

For some disorders where inhaled NO therapy has been administered in the past, the treatment has not been entirely effective, or has resulted in undesirable side effects. Increased levels of iron nitrosylhemoglobin and methemoglobin have been observed in some cases (Gladwin, M.T. *et al.*, *Proc. Natl. Acad. Sci. USA* 97:9943-9948, 2000; Deem, S. *et al.*, *Am. J. Respir. Crit. Care Med.* 163:1164-1170, 2001).

It has not been appreciated heretofore that SNO level within RBCs should be measured, and that an abnormal SNO level can be an indicator of a medical disorder. Therapies for the medical conditions and medical interventions described herein should be administered with the object of not only alleviating symptoms, as one would usually require for therapeutic effectiveness, but also with the object of bringing the patient's NO:Hb as SNO to a desired value or to within a range of values. With a measurable endpoint as a goal, it is then within the skill of one of ordinary skill in the art to choose an NO donor compound, for example, an S-nitrosothiol, a route of administration, and calculate dosages to reach this endpoint. Monitoring the patient's blood, with methods as illustrated in Examples 15 and 16, is useful in making any adjustments in the therapy to reach the desired endpoint. Thus, a therapeutically effective amount for the methods of therapy described herein is defined as an amount that will, in addition to alleviating symptoms characteristic of the particular condition (e.g., relief of angina associated with

coronary disease, relief of painful crisis in sickle cell disease, lowering of pulmonary artery pressure in pulmonary hypertension, lowering of systemic blood pressure in systemic hypertension), bring the mammal's (or human patient's) NO:Hb as SNO or total NO:Hb or SNO:iron nitrosylhemoglobin to a desired value, or to raise red blood  
5 cell SNO to a desired extent. Treatment of a patient to a subjective alleviation of symptoms, without bringing the red blood cells to an objectively-measured endpoint may be an incomplete treatment and leave the patient susceptible to further complications from deficiencies in oxygen delivery.

It is possible to choose a pharmaceutically active reagent, with the goal of  
10 altering red blood cell properties, to selectively increase SNO-Hb without substantially increasing iron nitrosylhemoglobin. Ethyl nitrite can be administered to a patient at 80 ppm, for example. Also see Example 17.

It has been shown that there is a great variation in normal levels of S-nitrosothiol content in red blood cells, and that isolated blood loses S-nitrosothiol content rapidly  
15 (Figure 13). SNO of the red blood cell is essential to providing biologically active NO for vasodilation, and other physiological effects. Therefore, blood to be transfused into patients should be treated to raise the NO:Hb in the blood, specifically by a method that will raise SNO content.

It is most preferred to raise SNO in red blood cells, by either *ex vivo* or *in vivo*  
20 methods, such that SNO is at least one-half of total NO, and that NO:Hb as SNO is at least as great as the mean value for normal humans.

Raising the NO:hemoglobin ratio to a desired level in isolated red blood cells, whether to a value within the normal range, to approximately the mean value for normal humans, to a value greater than the mean to produce a pharmacological or therapeutic  
25 effect, or to a value that is higher than the normal range, can be accomplished by *in vivo* or *ex vivo* treatment of red blood cells, for example by using whole blood from one or more donor, or from a patient who is to receive the blood. The *ex vivo* treatment of the red blood cells can be by incubating deoxygenated red blood cells with one or more S-

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nitrosothiols, as described in Examples 3 and 4, for example, or by incubating the red blood cells with an ester of an S-nitrosothiol, such as S-nitrosocysteine ethyl ester. The *ex vivo* treatment of the red blood cells can be by incubation of oxygenated red blood cells with NO gas dissolved in a buffer, for example, using 1  $\mu$ M NO (Gow, A.J. *et al.*,

5 *Proc. Natl. Acad. Sci. USA* 96:9027-0032,1999). The *ex vivo* treatment can also be  
incubation of the red blood cells with inorganic or organic nitrites, such as alkyl nitrites,  
e.g., ethyl nitrite in a liquid solution. Ethyl nitrite can be added to whole blood at a  
molar ratio of 1:1000 to 10:1 ethyl nitrite:Hb, for 30 seconds to 10 minutes. The treated  
blood cells (to have a NO:hemoglobin ratio of 1:25 to 1:1000) are administered to a  
10 patient, for example, intravenously.

Although S-nitrosoglutathione has not been observed to go through the red blood cell membrane, S-nitrosoglutathione is effective in transnitrosation reactions in which biologically active NO passes through the red blood cell membrane. See

Example 23. As described in Pawloski *et al.* (Pawloski, J.R. *et al.*, *Nature* 409:622-626, 2001), hemoglobin-derived S-nitrosothiol is found in association with the inside of the red blood cell membrane. S-nitrosoglutathione and possibly other S-nitrosothiols that have not been observed to pass through the red blood cell membrane may pass partially into the membrane, gaining access to cysteine thiols of the anion exchange protein AE1. Alternatively, S-nitrosothiols not passing through the membrane may participate in transnitrosation reactions with surface thiols. The reverse reaction is illustrated in Example 23.

A number of methods, relying on a variety of reagents, are now available to increase biologically active NO in isolated red blood cells. The methods can comprise a step of contacting the isolated red blood cells with a gas or liquid solution comprising

25 NO, or with a gas solution or a liquid solution comprising ethyl nitrite, or contacting the isolated red blood cells with a liquid solution comprising an S-nitrosothiol or an ester of an S-nitrosothiol.

Thiols within the red blood cell (glutathione as the most abundant) and in the plasma and in other body fluids facilitate the transfer of NO biological activity. Thus, a method in which a composition comprising a thiol is administered to a human, e.g., intravenously, is a treatment to potentiate the NO-mediated bioactivity of the red blood

5 cells. This method can be used for any of the disorders or conditions or medical interventions described herein characterized by a low NO condition or a low NO:Hb ratio. For example, glutathione or N-acetylcysteine can be administered IV (50-200 mg/kg) every 6 hours.

Stored isolated blood, as blood to be used in whole blood transfusions, can be

10 supplemented with a composition comprising one or more thiols, for the potentiation of NO-mediated activity of the red blood cells after they are introduced into a human. For example, glutathione or N-acetylcysteine can be added to the blood such that a dose of 1-20 grams of the thiol compound is administered in a transfusion. A dose can be formulated to administer 50-200 mg/kg in a standard bag of blood.

15 The term hemoglobin or Hb as used herein includes variant forms such as natural or artificial mutant forms differing by the addition, deletion and/or substitution of one or more contiguous or non-contiguous amino acid residues, or modified polypeptides in which one or more residues is modified, and mutants comprising one or more modified amino acid residues. Hb also includes chemically modified forms, as

20 well as genetically altered forms, such as fusion proteins, and truncated forms. It also includes Hbs of all species and variant forms thereof. The biological and/or chemical properties of these variant Hbs may be different from those of hemoglobins which are found naturally occurring in animals.

It will be appreciated that "biologically active NO" or "biological equivalents of

25 NO" exist in biological systems not only as nitric oxide (NO) gas, which can be put into a gas solution or liquid solution, but also as molecules that can donate or transfer NO in the form of NO or NO<sup>+</sup>. These molecules exist in various redox forms and as biologically active adducts of nitric oxide such as S-nitrosothiols, which can include S-

nitrosoproteins, S-nitroso-amino acids and other S-nitrosothiols such as S-nitrosoglutathione (Stamler, J.S. *Cell* 78:931-936 (1994)).

A blood substitute can be a biologically compatible liquid which performs one or more functions of naturally occurring blood found in a mammal such as oxygen carrying and/or delivery, NO carrying and/or delivery, and the scavenging of free radicals. A blood substitute can also comprise one or more components of such a liquid which, when infused into a mammal, perform one or more functions of naturally occurring blood. Examples of blood substitutes include compositions or preparations of various forms of hemoglobin. Such compositions or preparations can also include other biologically active components, such as a low molecular weight thiol or nitrosothiol.

The compounds and therapeutic preparations of this invention to be used in medical treatment are intended to be used in therapeutically effective amounts, in suitable compositions, which can be determined by one of skill in the art. Modes of administration are those known in the art which are most suitable to the affected site or system of the medical disorder. A preferred mode of administration of compositions comprising hemoglobins or loaded red blood cells is intravenous.

Suitable pharmaceutical carriers or vehicles can be combined with active ingredients employed in a therapeutic preparation, if necessary. It will be appreciated that the actual amounts of the active components in a specific case will vary according to the specific component being utilized, the particular composition formulated, the mode of administration and the age, weight and condition of the animal or human patient, for example. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

The present invention is more specifically illustrated in the following examples, which are not intended to be limiting in any way.

## EXAMPLES

Example 1: Interactions of NO and RSNO with Hb

It was observed that naturally occurring N-oxides, such as NO and RSNOs (Gaston, B., *et al.* (1993; Scharfstein, J.S., *et al.*, *J. Clin. Invest.* 94:1432-1439 (1994); 5 Clancy, R.M., *et al.*, *Proc. Natl. Acad. Sci. USA* 91:3680-3684 (1994)), differed markedly in their reactions with Hb. NO bound very rapidly to deoxyHb ( $\text{Hb}[\text{FeII}]$ ), forming relatively stable  $\text{Hb}[\text{FeII}]NO$  complexes (Figure 1A), and converted oxyHb ( $\text{Hb}[\text{FeII}]O_2$ ) to methemoglobin ( $\text{Hb}[\text{FeIII}]$ ) and nitrate (Figure 1B), confirming previous reports (Olson, J.S., *Methods in Enzymol.* 76:631-651 (1981); Toothill, C., 10 *Brit. J. Anaesthy.*, 39:405-412 (1967)). In contrast, RSNOs were found to participate in transnitrosation reactions with sulphydryl groups of Hb, forming S-nitrosohemoglobin (SNO-Hb), and did not react with the heme centers of either deoxyHb or  $\text{Hb}(\text{FeII})O_2$  (Figures 1C and 1D).

15 A. Interaction of NO with deoxyHb

Conversion of deoxyHb ( $\text{Hb}[\text{FeII}]$ ) to  $\text{Hb}(\text{FeII})NO$  is observed upon incubation of  $\text{Hb}(\text{FeII})$  with increasing concentrations of nitric oxide. (See Figure 1A.)

a. Deoxy Hb. b, c, d. Reaction mixtures of NO and  $\text{Hb}(\text{FeII})$  in ratios of 1:1, 2:1 and 10:1, respectively. The reaction product  $\text{Hb}(\text{FeII})NO$  formed essentially instantaneously 20 on addition of NO (*i.e.*, within instrument dead time).

B. Interaction of NO with oxyHb

Conversion of oxyHb ( $\text{Hb}[\text{FeII}]O_2$ ) to metHb ( $\text{HbFeIII}$ ) is observed upon incubation of oxyHb with increasing concentrations of NO. (See Figure 1B.) a. oxy Hb. b, c, d. Reaction mixtures containing NO and oxyHb in ratios of 1:1, 2:1 and 10:1, 25 respectively. Methemoglobin formation occurred instantaneously on addition of NO (*i.e.*, within instrument dead time).

C. Interaction of S-nitrosothiols with deoxyHb

Conversion of Hb(FeII) to SNO-Hb(FeII) is observed upon incubation of either GSNO (shown) or S-nitrosocysteine (CYSNO) with deoxy Hb. There is little (if any) interaction of RSNO with the heme functionalities of Hb. (See Figure 1C.) *a.*

5 deoxyHb. *b, c, d.* Reaction mixtures of GSNO and Hb(FeII) in ratios of 1:1, 2:1 and 10:1, respectively. Spectra were taken after 60 min of incubation in *b, c,* and 15 min in *d.* Further analysis of reaction products revealed the formation of moderate amounts of SNO-Hb in all cases. Yields of SNO-Hb (S-NO/Hb) in *b, c,* and *d* at 60 min were 2.5%, 5% and 18.5%, respectively. (See Figure 1D and Figure 2A.)

10 D. Interaction of S-nitrosothiols with oxyHb

Conversion of Hb(FeII) $O_2$  to SNO-Hb(FeII) $O_2$  is observed upon incubation of either GSNO (shown) or CYSNO with oxyHb. There is little (if any) reaction of GSNO (or CYSNO) at the heme centers of Hb(FeII) $O_2$ . Specifically, the capacity for  $O_2$  binding to heme is unaffected by RSNOs. (See Figure 1D.) *a.* oxyHb. *b, c, d.* Reaction mixtures of GSNO and oxyHb in ratios of 1:1, 2:1, and 10:1, respectively. Spectra were taken after 60 min of incubation in the spectrophotometer. Further analysis of reaction products revealed the formation of SNO-Hb in all cases. Yields of SNO-Hb in spectra *b, c* and *d* were 5%, 10% and 50% (S-NO/Hb), respectively. In 5 other determinations, the yield of S-NO/Hb was  $0.37 \pm 0.06$  using GSNO (pH 7.4, 10-fold excess over Hb)

15 and  $\sim 2$  SNO/tetramer ( $1.97 \pm 0.06$ ) using CYSNO (*vida infra*). These last data are in agreement with reports that human HbA contains 2 titratable SH groups.

20

Methods

Human HbA<sub>o</sub> was purified from red cells as previously described (Kilbourn, R.G., *et al.*, *Biochem. Biophys. Res. Comm.* 199:155-162 (1994)). Nitric oxide solutions 25 were rigorously degassed and purified according to standard procedure (Beckman, J. S.,

*et al.*, *Methods in Nitric Oxide Research*, Feilisch and Stamler, eds., Wiley Chichester, U.K. (1996)) and saturated solutions were transferred in air tight syringes.

Deoxygenation of Hb was achieved by addition of excess dithionite (NO studies) or by reduction of Hb(FeII)O<sub>2</sub> through evacuation in Thunberg tubes (RSNO studies; as

5 RSNOs react with dithionite). RSNOs were synthesized as previously described (Gaston, B., *et al.*, (1993); Arnelle and Stamler, *Arch. Biochem. Biophys.* 318:279-285 (1995)). Incubations with HbA<sub>0</sub> were made in phosphate buffer, pH 7.4, 0.5 mM EDTA. Quantifications of SNO-Hb were made according to the method of Saville (Gaston, B., *et al.*, (1993); Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 90:444-448 10 (1992)) after purification of protein with Sephadex G-25 columns. The Saville method, which assays free NO<sub>x</sub> in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore N-(naphthyl)ethylenediamine. No low molecular weight S-NO complexes survived this purification and all activity was protein precipitable. The reactions and spectra were carried out using a Perkin Elmer UV/Vis 15 Spectrometer, Lambda 2S.

Example 2: Allosteric Function of O<sub>2</sub> in Regulation of Hb S-nitrosylation

Oxygenation of Hb is associated with conformational changes that increase the reactivity of cys $\beta$ 93 to alkylating reagents (Garel, C., *et al.*, *J. Biochem.*, 123:513-519 (1982); Jocelyn, P.C., *Biochemistry of the SH Group*, Academic Press, London, p. 243 20 (1972); Craescu, C.T., *et al.*, *J. Biol. Chem.*, 261:14710-14716 (1986)). The physiological importance of this effect has not been explained previously. We observed that rates of S-nitrosylation of Hb were markedly dependent on conformational state. In the oxy conformation (R state), S-nitrosylation was more rapid than in the deoxy conformation (T state) (Figure 2A). The rate of S-nitrosylation was accelerated in both 25 conformations by alkaline conditions (*i.e.*, rate at pH 9.2>pH 7.4), which would tend to expose the cys $\beta$ 93 that is otherwise screened from reaction by the C-terminal histidine 146 $\beta$ . The salt bridge (asp  $\beta$ 94 --- his  $\beta$ 146) tying down the histidine residue is

loosened at high pH. These data suggest that the increase in thiol reactivity associated with the R state derives, at least in part, from improved NO access rather than a conformation-induced change in pK.

A. Oxygenation accelerates S-nitrosylation of Hb

5 Rates of Hb S-nitrosylation by S-nitrosocysteine (CYSNO) are faster in the oxy conformation ( $\text{Hb}[\text{FeII}]\text{O}_2$ ) than in the deoxy state ( $\text{Hb}[\text{FeII}]$ ).

Methods

10 Incubations were performed using 10-fold excess CYSNO over protein (50  $\mu\text{M}$ ) in aerated 2% borate, 0.5 mM EDTA (oxyHb), or in a tonometer after rapid  $\text{O}_2$  evacuation (deoxyHb). At times shown in Figure 2A, samples were rapidly desalted across G-25 columns (preequilibrated with phosphate buffered saline, 0.5 mM EDTA, pH 7.4) to remove CYSNO, and analyzed for SNO-Hb by the method of Saville (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)).

B. Deoxygenation accelerates denitrosylation of Hb

15 Rates of RSNO decomposition (and transfer) are much faster in the deoxy conformation [ $\text{SNO-Hb(FeII)}$ ] than in the oxy state [ $\text{SNO-Hb(FeII)}\text{O}_2$ ]. The decomposition of SNO-Hb(FeII) is further accelerated by the presence of excess glutathione. Within the dead time of our measurements (~15 sec) a major fraction of SNO-Hb(FeII) was converted to GSNO.

20 Methods

Hbs in PBS (0.5 mM EDTA, pH 7.4) were incubated in air (oxy) or in a tonometer previously evacuated of  $\text{O}_2$  (deoxy). SNO-Hb(FeII) $\text{O}_2$  decomposition was determined by the method of Saville (Saville, B., *Analyst*, 83:670-672 (1958)). Spontaneous decomposition of SNO-Hb(FeII) was followed spectrophotometrically by

formation of Hb(FeII)NO. Transnitrosation reactions with glutathione were performed by addition of 100-fold excess glutathione over protein (50  $\mu$ M), immediate processing of the reaction mixture under anaerobic conditions followed by rapid TCA precipitation, and analysis of RSNO in the supernatant. Rates of NO group transfer were too rapid to  
5 measure accurately by the standard methods used in this study.

Example 3: NO-related Interactions with Cysteine Residues of Hb in Physiological Systems

Given that Hb is largely contained within red blood cells, potential mechanisms by which S-nitrosylation of intracellular protein might occur were explored. Incubation  
10 of oxygenated rat red blood cells with S-nitrosocysteine resulted in very rapid formation of intracellular SNO-Hb(FeII) $O_2$  (Figure 3A). Rapid oxidation of Hb was not observed under these conditions. Intraerythrocytic SNO-Hb also formed when red blood cells were treated with S-nitrosohomocysteine or S-nitrosocysteinylglycine, but not with S-nitrosoglutathione (GSNO). Thus, erythrocyte access of RSNOs is thiol group specific.  
15 Exposure of oxygenated red blood cells to NO resulted primarily in metHb formation.

Endothelium-derived relaxing factor (EDRF) and hemoglobin

Hb-mediated inhibition of endothelium-dependent relaxations is commonly used as a marker of NO responses. Inasmuch as reactions with either metal or thiol centers of Hb should lead to attenuated NO/EDRF (endothelium-derived relaxing factor)  
20 responses, we attempted to elucidate the molecular basis of inhibition. Hb preparations in which  $\beta$ 93 thiol groups had been blocked with N-ethylmaleimide (NEM) or the hemes blocked by cyanmet (FeIIICN)-derivitization were studied in an aortic ring bioassay, and their activities compared with that of native Hb. Both cyanmet-Hb and NEM-Hb caused increases in vessel tone and attenuated acetylcholine (EDRF)-mediated  
25 relaxations (Figure 3B). However, native Hb was significantly more effective than either of the modified Hb preparations (Figure 3B). Taken in aggregate, these studies

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suggest that both the thiol and metal groups of Hb contribute to its NO-related activity. To verify formation of an S-nitrosothiol in Hb, we established a bioassay in which 2 cm segments of thoracic aorta were interposed in Tygon tubing, through which 3 cc of Krebs solution containing Hb (4  $\mu$ M) and ACh (2  $\mu$ M) were circulated by roller pump 5 (1.5 cc/min x 5 min). Analysis of the effluent (Gaston, B., *et al.*, (1993)) revealed the formation of SNO-Hb ( $20 \pm 4$  nM) in 5 of 5 experiments.

#### A. S-nitrosylation of intraerythrocytic Hb

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5 mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular SNO-10 Hb(FeII)O<sub>2</sub>. MethHb does not form rapidly. Separation of intracellular RSNOs across G-25 columns reveals that only a small percentage exists as low molecular weight S-nitrosothiol (e.g. GSNO) at most time points. By 60 min, 3 of the 4 available SH 15 groups of Hb are S-nitrosylated (note that rat Hb contains 4 reactive SH groups). (See Figure 3A.) Inset shows spectra of SNO-Hb isolated from rat erythrocytes and related analyses. Spectrum *A* is that of SNO-Hb isolated from erythrocytes following G-25 chromatography. Treatment of *A* with dithionite results in reduction of the S-NO moiety, liberating free NO which is autocaptured by deoxy Hb, forming Hb(FeII)NO (note that dithionite simultaneously deoxygenates Hb) (spectrum *C*). This spectrum (*C*) 20 reveals a stoichiometry of ~3 S-NOs per tetramer. The spectrum of Hb(FeII)NO containing 4 NO's per tetramer is shown for comparison (inset, spectrum *B*).

#### Methods

At shown intervals, red blood cells were pelleted rapidly by centrifugation, washed three times, lysed in deionized water at 4°C, and the cytosolic fraction subjected 25 to rapid desalting across G-25 columns. Intracellular SNO-Hb was measured by the method of Saville (Gaston, B., *et al.*, (1992); Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci.*

USA, 89:444-448 (1992)), and confirmed spectroscopically (inset of Figure 3A) as described above.

B. Molecular Basis of EDRF/Hb Interaction.

The effects of native Hb on EDRF responses were compared with Hb preparations in which the thiol or heme centers had been blocked by alkylation or cyanmet derivitization, respectively. All preparations of Hb elicited contractions; however, those of native Hb (in which both SH and metal centers are free for interaction) were most pronounced. (See Figure 3B.) Likewise, acetylcholine (ACh) mediated relaxations were most effectively inhibited by native Hb. Relaxations were inhibited to lesser degrees by cyanmet Hb (CN-Hb)(in which hemes were blocked from reaction) and NEM-Hb (in which thiol groups were alkylated by N-ethylmaleimide). See Table 1. These data illustrate that both heme and  $\beta$ 93SH groups of Hb contribute to reversal of EDRF responses. Direct measurement of SNO-Hb, formed from EDRF under similar conditions, is described in Example 8.

15 Methods

Descending rabbit thoracic aorta were cut into 3 mm rings and mounted on stirrups attached to force transducers (model FT03, Grass Instruments, Quincy, MA) for measurement of isometric tone. The details of this bioassay system have been previously described (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)). Cyanmet Hb was prepared from human HbA according to published protocols (Kilbourn, R.G. et al. *Biochem. Biophys. Res. Comm.* 199:155-162, (1994)). Alkylation of HbA with N-ethylmaleimide was followed by desalting across G-25 Sephadex to remove excess NEM. Removal of unmodified Hbcys $\beta$ 93 was achieved by passage through Hg-containing affinity columns. The alkylation of free SH groups was verified 25 using 5,5'-dithio-bis[2-nitrobenzoic acid].

TABLE 1

ADDITIONS	% INCREASE IN TENSION	% ACh RELAXATION
Hb (1 $\mu$ M)	40.8 $\pm$ 2.3 (n=7)	31.9 $\pm$ 6.9 (n=7)
NEM-Hb (1 $\mu$ M)	29.4 $\pm$ 1.3** (n=7)	60.5 $\pm$ 3.9* (n=7)
CN-Hb (1 $\mu$ M)	12.9 $\pm$ 3.0** (n=6)	80.7 $\pm$ 1.0** † (n=4)
ACh (1 $\mu$ M)		98.3 $\pm$ 0.6 (n=10)

\* P<0.01; \*\* P<0.001, Compared to Hb; † P<0.001, Compared to ACh

**Example 4: Transduction of SNO-Hb Vasoactivity**

Arterial red blood cells contain two physiologically important forms of hemoglobin: Hb(FeII) $O_2$  and Hb(FeIII) (Antonini *et al.* (1971)). Arterial-venous differences in the S-nitrosothiol content of intraerythrocytic Hb suggest that the NO group is released during red cell transit. Such findings raise the possibility of functional consequences, perhaps influenced by the redox state of heme and its occupation by ligand. SNO-Hb(FeII) $O_2$  was found to possess modest NO-like activity when tested in a vascular ring bioassay. Specifically, the contraction elicited by SNO-Hb(FeII) $O_2$  was less than that of native Hb(FeII) $O_2$ , indicating that S-nitrosylation partially reverses the contractile effects of Hb (Figure 4A). By comparison, SNO-Hb(FeIII) was found to be a vasodilator (Figure 4A). Notably, free NO was devoid of relaxant activity in the presence of Hb(FeII) $O_2$  or Hb(FeIII) (not shown).

Red blood cells contain millimolar concentrations of glutathione. As equilibria among RSNOs are rapidly established through RSNO/thiol exchange (Arnelle and Stamler, J.S., *Arch. Biochem. Biophys.*, 318:279-285 (1995)), the vasoactivity of SNO-Hb was reassessed in the presence of glutathione. Figure 4B illustrates that glutathione potentiated the vasodilator activity of both SNO-Hb(FeII) $O_2$  and SNO-Hb(FeIII). GSNO formation under these conditions (confirmed chemically and in bioassay experiments) appeared to fully account for this effect. Further kinetic analyses revealed that transnitrosation involving glutathione was more strongly favored in the equilibrium with SNO-Hb(FeIII) than SNO-Hb(FeII) $O_2$  (Figure 4C). Given the findings of steady-state levels of SNO-Hb in red blood cells (Table 2 and Figure 3A), these results suggest that 1) the equilibrium between naturally occurring RSNOs and Hb(cys $\beta$ 93) lies toward SNO-Hb under physiological conditions; 2) that transnitrosation reactions involving SNO-Hb and GSH are likely to occur within red blood cells (the presence of low molecular weight RSNOs in erythrocytes loaded with SNO-Hb has been verified in studies herein); and 3) that oxidation of the metal center of Hb will shift the equilibrium toward GSNO, thereby potentially influencing bioactivity.

Additional mechanisms of NO group release from SNO-Hb were sought. Arterial-venous differences in levels of SNO-Hb raised the possibility that S-NO bond stability may be regulated by the changes in Hb conformation accompanying deoxygenation. To test this possibility, we compared the rates of NO group release 5 from SNO-Hb(FeII) $O_2$  and SNO-Hb(FeIII). Deoxygenation was found to enhance the rate of SNO-Hb decomposition (Figure 2B). These rates were accelerated greatly by glutathione in a reaction yielding GSNO (Figure 2B). Our results illustrate that  $O_2$ -metal interactions influence S-NO affinity, and suggest a new allosteric function for Hb.

For SNO-Hb to be of physiological importance, it must transduce its NO-related 10 activity across the erythrocyte membrane. We therefore explored this possibility by incubating erythrocytes containing SNO-Hb in physiologic buffer, and measuring the accumulation of extracellular RSNOs over time. Figure 4D illustrates that red blood cells export low molecular weight (trichloroacetic acid soluble) S-nitrosothiols under these conditions. Importantly, the degree of hemolysis in these experiments was trivial 15 (<0.5%), and correction for lysis did not significantly impact on rates of RSNO release. These results establish that an equilibrium exists between low molecular weight and protein RSNOs within the red cell, and that intracellular location is unlikely to be a limiting factor in the transduction of such NO-related activity to the vessel wall.

#### A. Concentration-effect responses of different SNO-Hb preparations

20 Contractile effects of Hb(FeII) $O_2$ (▲) are shown to be partially reversed by S-nitrosylation (SNO-Hb[FeII] $O_2$ (■); P = 0.02 by ANOVA vs Hb(FeII) $O_2$ ). (See Figure 4A.) Oxidation of the metal center of SNO-Hb (SNO-Hb[FeIII](●)) converts the protein into a vasodilator (P < 0.0001 by ANOVA vs SNO-Hb[FeII] $O_2$ ), with potency comparable to that of other S-nitrosoproteins (Stamler, J.S., et al., *Proc. Natl. Acad. Sci.* 25 *USA* 89:444-448 (1992)). The contractile properties of Hb(FeIII) are shown for comparison (□); n = 6-17 for each data point.

### Methods

Details of the vessel ring bioassay have been published (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444-448 (1992)). SNO-Hb(FeII)O<sub>2</sub> preparations were synthesized with 10-fold excess S-nitrosocysteine (CYSNO) over Hb(FeII)O<sub>2</sub> protein  
5 (2% borate, 0.5 mM EDTA, ~15 min incubation), after which desalting was performed across Sephadex G-25 columns. CYSNO was synthesized in 0.5 N HCl, 0.5 mM EDTA and then neutralized (1:1) in 1 M phosphate buffer containing 0.5 mM EDTA. SNO-Hb(FeIII) preparations followed a similar protocol, but used Hb(FeIII) as starting material. The latter was synthesized by treatment of Hb(FeII)O<sub>2</sub> with excess  
10 ferricyanide, followed by desalting across G-25 columns. SNO-Hb concentrations were verified spectroscopically and the S-nitrosothiol content was determined by the method of Saville (Stamler, J.S., *et al.*, *Proc. Nat. Acad. Sci. USA* 89:444-448 (1992)). The S-NO/tetramer stoichiometry for both SNO-Hb preparations was ~2. Oxidation of the heme was undetectable by uv-spectrophotometric methods.  
15

#### B. Potentiation of SNO-Hb effects by glutathione

Addition of glutathione (100 μM) to bioassay chambers potentiates the dose-response to both SNO-Hb(FeII)O<sub>2</sub>(■) and SNO-Hb(FeIII)(●) (See Figure 4B. n=6-12; p<0.0001 for both by ANOVA, compared with the respective tracings in Figure 4A). Glutathione had a transient affect on baseline tone in some experiments, and did not  
20 significantly influence the response to Hb(FeII)O<sub>2</sub> (▲).

#### C. Transnitrosation between SNO-Hb and glutathione

Rates of NO group transfer from SNO-Hb (100 μM) to glutathione (10 mM) are displayed for SNO-Hb(FeII)O<sub>2</sub> (oxy) and SNO-Hb(FeIII) (met) (n=5). Data are presented as the amount of GSNO formed relative to the starting SNO-Hb  
25 concentration. The transfer is more rapid for SNO-Hb(FeIII) than SNO-Hb(FeII)O<sub>2</sub>.

( $p < 0.002$  by ANOVA), suggesting that the GSNO/SNO-Hb equilibrium is shifted toward GSNO by formation of met Hb.

#### Methods

Thiol/SNO-Hb exchange, forming GSNO, was verified chemically (Stamler, J.S., *et al.*, *Proc. Natl Acad. Sci. USA* 89:444-448 (1992)) following trichloroacetic acid precipitation ( $n=5$ ). These results were verified in separate experiments by measuring the residual SNO-Hb concentration, following separation of reaction mixtures across G-25 columns.

#### D. Export of S-nitrosothiols by red blood cells

Human red blood cells containing SNO-Hb are shown to export low molecular weight RSNOs over time. Hemolysis, which ranged from 0-<0.5% over one hour and did not correlate with rates of RSNO release, could account for only a trivial fraction of the measured extracellular RSNO.

#### Methods

Packed human red blood cells were obtained by centrifugation, washed, and resuspended in phosphate buffered saline containing 5 mM SNOCYS (0.5 mM EDTA, pH 7.4) for one hour. This results in a red cell preparation containing SNO-Hb (FeII $O_2$ /FeIII mixture) with a stoichiometry of 0.5 S-NO/tetramer. The red blood cells were then washed repeatedly to remove residual CYSNO (verified), and incubated in Krebs' solution (1:4). The accumulation of extracellular RSNO was measured over time by the method of Saville (Saville, B., *Analyst*, 83:670-672 (1958)). Hemolysis was determined by spectral analysis of red blood cell supernatants following centrifugation.

**Example 5: SNO-Hb Bioactivity *In Vivo***

Systemic administration of cell-free Hb results in hypertensive responses which have been attributed to NO scavenging by the heme (Vogel, W.M., *et al.*, *Am. J. Physiol.* 251:H413-H420 (1986); Olsen, S.B., *et al.*, *Circulation* 93:329-332 (1996)).

5 To determine if SNO-Hb is free of this adverse affect, and to explore if *in vitro* mechanisms of NO release extend to the *in vivo* circumstance, we compared responses to Hb and SNO-Hb infused as a bolus into the femoral vein of anesthetized rats. As illustrated in Figure 5, Hb(FeII)O<sub>2</sub> (200 nmol/kg) caused an increase in mean arterial pressure of  $20 \pm 3$  mm Hg ( $n = 4$ ;  $P < 0.05$ ). In contrast, SNO-Hb(FeII)O<sub>2</sub> did not

10 exhibit hypertensive effects and SNO-Hb(FeIII) elicited hypotensive responses (Figure 5). Thus, the profiles of these compounds *in vivo* closely resemble those seen *in vitro* (Figure 4A). Moreover, to demonstrate that the physiological responses of red cells are comparable to those of cell-free Hb preparations, erythrocytes containing SNO-Hb were injected into the femoral vein of rats pretreated with L-NMMA (50 mg/kg) to deplete

15 endogenous RSNOs. At levels of SNO-Hb comparable to those found in the normal rat (0.1-0.5  $\mu$ M), SNO-Hb containing red blood cells elicited hypotensive responses ( $8 \pm 1$  mm Hg; mean  $\pm$  SEM;  $n=9$ ), whereas native (SNO-Hb depleted) red blood cells did not ( $P=0.001$ ). These changes in mean blood pressure of ~10% are on the order of those that differentiate normotension from hypertension in man, and in the therapeutic range

20 of some antihypertensive regimens. The effects of both Hb and SNO-Hb -- whether cell-free or contained within red cells -- were transient, suggesting that S-nitrosylation of Hb and metabolism of SNO-Hb is occurring *in vivo*, with consequent restoration of blood pressure. The bioactivity of SNO-Hb in blood, where S-NO/heme stoichiometries approach 1:50,000, is a dramatic illustration of the resistance of this NO-related activity

25 to Hb(Fe) inactivation.

### In vivo effects of cell-free Hb and SNO-Hbs

Administration of 2-200 nmol/kg Hb(FeII)O<sub>2</sub> (as a bolus) into the femoral vein of a Sprague-Dawley rat is shown to increase mean arterial pressure in a dose-dependent manner. At 200 nmol/kg, mean arterial pressure increased by 25 mm Hg (20 ± 3 mm Hg; n=4; P < 0.05). Elevations in blood pressure reversed within 10-15 min. SNO-Hb(FeII)O<sub>2</sub> infusions (over the same dose range) are shown to ameliorate Hb(FeII)O<sub>2</sub>-induced hypertension without causing overt changes in blood pressure. A similar response was seen at higher doses. By comparison, SNO-Hb(FeIII) infusions caused a significant fall in mean arterial pressure (pre 108 ± 4 mm Hg; post 74 ± 6 mm Hg, n = 5; P < 0.05) at the highest dose (200 nmol/kg). Hypotensive responses tended to be transient with blood pressure normalizing over 10 minutes. A fall in blood pressure was also seen with injection of erythrocytes containing SNO-Hb.

### Methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Codas) was used for data acquisition.

### Example 6: Loading of Red Blood Cells With S-Nitrosothiols

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5 mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular S-nitrosothiols. MetHb does not form rapidly. Separation of cell content across G-25 columns establishes the formation of intraerythrocytic low molecular weight S-nitrosothiol, e.g. S-nitrosoglutathione (GSNO). By 2 min., one can achieve as much as millimolar GSNO.

Method for assay of RSNO

S-nitrosocysteine (5 mM) treated red blood cells are pelleted rapidly by centrifugation, washed three times, lysed in deionized water at 4°C, and the cytosolic fraction subjected to rapid desalting across G-25 columns. Intracellular RSNO is measured by the method of Saville and can be confirmed spectroscopically.

Effects on blood pressure from loaded red blood cells

Red blood cells treated with S-nitrosocysteine (to produce SNO-RBCs) and introduced into the femoral vein of a Sprague-Dawley rat decreased mean arterial pressure in a dose-dependent manner. For red blood cells in which SNO-Hb was assayed at 0.3  $\mu$ M (the endogenous *in vivo* SNO-Hb concentration), arterial pressure decreased by  $8 \pm 1$  mm Hg (mean  $\pm$  SEM for 9 experiments;  $p < 0.001$  compared to untreated red blood cell controls). For red blood cells in which SNO-Hb was assayed at 0.5  $\mu$ M, arterial pressure decreased by 10 mm Hg. For red blood cells in which SNO-Hb was assayed at 0.1  $\mu$ M (a sub-endogenous SNO-Hb concentration), arterial pressure decreased by 6 mm Hg. The administration of untreated red blood cells caused no effect or a slight increase in arterial blood pressure. Administration of L-monomethyl-L-arginine (L-NMMA; 50 mg/kg) caused an increase in blood pressure of about 20 mm Hg. Changes in blood pressure from a bolus administration of loaded red blood cells lasted 15-20 minutes.

15 Further methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Codas) was used for data acquisition.

Example 7: Effects of SNO-Hb on Coronary Vasodilation, Coronary Flow and Blood Pressure

SNO-Hb was synthesized as described in Example 4A. Completion of the reaction was determined as described in Example 4A. Twenty-four healthy mongrel 5 dogs (25-30 kg) were anesthetized with intravenous thiamylal sodium (60-80 mg/kg) and subjected to left thoracotomy in the fourth intercostal space. The left circumflex coronary artery distal to the left atrial appendage was minimally dissected. A pair of 7-MHz piezoelectric crystals (1.5 X 2.5 mm, 15-20 mg) was attached to a Dacron backing and sutured to the adventitia on opposite surfaces of the dissected vessel segment with 10 6-0 prolene. Oscilloscope monitoring and on-line sonomicrometry (sonomicrometer 120-2, Triton Technology, San Diego, CA) were used to ensure proper crystal position. A pulse Doppler flow probe (10 MHz, cuff type) was implanted distal to the crystals. An inflatable balloon occluder was also placed distal to the flow probe. All branches of the circumflex artery between the crystals and the occluder were ligated. Heparin 15 sodium-filled polyvinyl catheters were inserted into the left ventricular cavity via the apex, into the left atrium via the atrial appendage, and into the ascending aorta via the left internal thoracic artery. The catheters, tubing, and wires were tunnelled to a subcutaneous pouch at the base of the neck.

After a 10 to 15 day recovery period, the catheters and wires were exteriorized 20 under general anesthesia, and 2-3 days later, each dog was given a bolus injection of SNO-Hb (0.4 mg) to evaluate vascular response. Two dogs that demonstrated <5% dilation of epicardial coronary vessels were excluded from subsequent studies, and two were excluded because of other technical reasons.

Dogs were trained and studied while loosely restrained and lying awake in the 25 lateral recumbent position. The laboratory was kept dimly illuminated and quiet. Aortic pressure, left ventricular end-diastolic pressure dP/dt external coronary diameter and coronary flow were monitored continuously. In 10 dogs, 0.1 ml of SNO-Hb solution, 50 nM/kg, was injected via the left atrial catheter. To verify potential effects

of solvent on vasculature, 0.1 ml injections of 30% ethanol in distilled water were given as vehicle control. Between injections, phasic coronary blood flow and coronary artery diameter were allowed to return to preinjection levels (minimum 15 minutes). Allowing a 15 minute period between injections resulted in no modification of repeated dose  
5 injections. To assess the direct and potential flow mediated indirect vasodilation effects of SNO-Hb on the conductance vessels, the dose was repeated in 6 of 10 dogs with partial inflation of the adjustable occluder to maintain coronary blood flow at or slightly below preinjection levels. The response to acetylcholine chloride (Sigma Chemical) was assessed in another group of 10 dogs following a similar protocol to that used for  
10 SNO-Hb.

Epicardial coronary diameter, coronary blood flow, heart rate, and aortic and left ventricular end-diagnostic pressures were compared before and after each SNO-Hb injection. The maximum changes in coronary dimension and blood flow were expressed as a function of increasing doses of SNO-Hb. The response of coronary  
15 dimension to increasing doses followed a characteristic sigmoid dose-response curve that could be described by the following equation

$$\text{Effect} = \frac{\text{maximal effect} \times \text{dose}}{K_D + \text{dose}}$$

where  $K_D$  is the drug-receptor complex dissociation constant and is the dose at which 50% of the maximum response ( $EC_{50}$ ) is achieved. In each animal, a nonlinear least-squares regression ( $r^2 > 0.90$ ) was performed on the dose-response data. The regression  
20 was constrained to the above equation. From the regression, values for maximum response and  $K_D$  were obtained for each individual animal. The mean of these values was then calculated to obtain an average  $K_D$  and maximum response for the study group. These values were used to generate a mean curve, which was plotted with the mean dose-response values. (See Figures 6A-6F.)

Example 8: Endogenous Levels of S-nitrosohemoglobin and Nitrosyl(FeII)-Hemoglobin in Blood

To determine if SNO-Hb is naturally occurring in the blood, and if so, its relationship to the O<sub>2</sub> transport capacity and nitrosylated-heme content of red cells, we developed an analytical approach to assay the S-nitrosothiol and nitrosyl-heme content of erythrocytes (Table 2). Arterial blood was obtained from the left ventricle of anesthetized rats by direct puncture and venous blood was obtained from the jugular vein and inferior vena cava. Hb was then purified from red cells and assayed for RSNO and (FeII)NO content. Arterial blood contained significant levels of SNO-Hb, whereas levels were virtually undetectable in venous blood (Table 2). Measurements made 45 minutes after infusion of the NO synthase inhibitor N<sup>ω</sup>-monomethyl-L-arginine (L-NMMA) (50 mg/kg), showed a depletion of SNO-Hb as well as total Hb-NO (82 and 50 ± 18%, respectively; n=3-5; p<0.05). These data establish the endogenous origin of SNO-Hb, although some environmental contribution is not excluded. The arterial-venous distribution seen for SNO-Hb was reversed in the case of Hb(FeII)NO, which was detected in higher concentrations in partially deoxygenated (venous) erythrocytes (Table 2). Accordingly, the proportion of nitrosylated protein thiol and heme appears to depend on the oxygenation state of the blood. Consistent with these findings, Wennmalm and coworkers have shown that Hb(FeII)NO forms mainly in venous (partially deoxygenated) blood (Wennmalm, A., et al., *Br. J. Pharmacol.*, 106(3):507-508 (1992)). However, levels of Hb(FeII)NO *in vivo* are typically too low to be detected (by EPR) and SNO-Hb is EPR-silent (*i.e.* it is not paramagnetic). Thus, photolysis-chemiluminescence represents an important technological advance, as it is the first methodology capable of making quantitative and functional assessments of NO binding to Hb under normal physiological conditions.

Table 2

Endogenous Levels of S-Nitrosohemoglobin and  
Nitrosyl(FeII)-Hemoglobin in Blood

5	Site	SNO-Hb (nM)	Hb(FeI)NO (nm)
	Arterial	311 ± 55*	536 ± 99 †
	Venous	32 ± 14	894 ± 126

\*P < 0.05 vs venous; † P < 0.05 for paired samples vs venous

Methods

10       Blood was obtained from the left ventricle (arterial) and jugular vein (venous) of anesthetized Sprague-Dawley rats. Comparable venous values were obtained in blood from the inferior vena cava. Red blood cells were isolated by centrifugation at 800 g, washed three times in phosphate buffered saline at 4°C, lysed by the addition of 4-fold excess volume of deionized water containing 0.5 mM EDTA, and desalted rapidly across G-25 columns according to the method of Penefsky at 4°C. In 24 rats, Hb samples were divided in two aliquots which were then treated or not treated with 10-fold excess HgCl<sub>2</sub> over protein concentration as measured by the method of Bradford. Determinations of SNO-Hb and Hb(FeII)NO were made by photolysis-chemiluminescence as described below. In 12 additional rats, further verification of the presence of SNO-Hb was made by assaying for nitrite after HgCl<sub>2</sub> treatment. Specifically, samples (with and without HgCl<sub>2</sub>) were separated across Amicon-3 (Centricon filters, m.w. cut off 3,000) at 4°C for 1 h, and the low molecular weight fractions collected in airtight syringes containing 1 µM glutathione in 0.5 N HCl. Under these conditions, any nitrite present was converted to S-nitrosoglutathione, which was

then measured by photolysis-chemiluminescence (detection limit ~1 nM). SNO-Hb was present in all arterial samples, and levels determined by this method ( $286 \pm 33$  nM) were virtually identical to and not statistically different from those shown in Table 2. In venous blood, SNO-Hb was undetectable ( $0.00 \pm 25$  nM); levels were not statistically different from those given above.

Method for assay of S-nitrosohemoglobin

A highly sensitive photolysis-chemiluminescence methodology was employed. A somewhat similar assay has been used for measuring RSNOs (S-nitrosothiols) in biological systems (Gaston, B., *et al.*, (1993); Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA* 89:7674-7677 (1992)). The method involves photolytic liberation of NO from the thiol, which is then detected in a chemiluminescence spectrometer by reaction with ozone. The same principle of operation can be used to cleave (and measure) NO from nitrosyl-metal compounds (Antonini, E. Brunori, M. *In Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). With adjustment of flow rates in the photolysis cell, complete photolysis of the NO ligand of Hb(FeII)NO could be achieved. Standard curves derived from synthetic preparations of SNO-Hb, Hb(FeII)NO, and S-nitrosoglutathione were linear ( $R > 0.99$ ), virtually superimposable, and revealing of sensitivity limits of approximately 1 nM. Two analytical criteria were then found to reliably distinguish SNO-Hb from Hb(FeII)NO: 1) signals from SNO-Hb were eliminated by pretreatment of samples with 10-fold excess  $HgCl_2$ , while Hb(FeII)NO was resistant to mercury challenge; and 2) treatment of SNO-Hb with  $HgCl_2$  produced nitrite (by standard Griess reactions) in quantitative yields, whereas similar treatment of Hb(FeII)NO did not. UV/VIS spectroscopy confirmed that NO remained attached to heme in the presence of excess  $HgCl_2$ .

Example 9: Oxygen-Dependent Vasoactivity of S-Nitrosohemoglobin: Contraction of Blood Vessels in R-Structure and Dilation in T-Structure

The details of this bioassay system have been published (Osborne, J.A., *et al.*, *J. Clin. Invest.* 83:465-473 (1989)). In brief, New Zealand White female rabbits weighing 5 3-4 kg were anesthetized with sodium pentobarbital (30 mg/kg). Descending thoracic aorta were isolated, the vessels were cleaned of adherent tissue, and the endothelium was removed by gentle rubbing with a cotton-tipped applicator inserted into the lumen. The vessels were cut into 5-mm rings and mounted on stirrups connected to transducers (model TO3C, Grass Instruments, Quincy, MA) by which changes in isometric tension 10 were recorded. Vessel rings were suspended in 7 ml of oxygenated Kreb's buffer (pH 7.5) at 37°C and sustained contractions were induced with 1 µM norepinephrine.

Best attempts were made to achieve equivalent baseline tone across the range of oxygen concentrations; i.e., hypoxic vessels were contracted with excess phenylephrine. Oxygen tension was measured continuously using O<sub>2</sub> microelectrodes (Model 733 Mini; 15 Diamond General Co., MI) (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981); Dewhirst, M.W. *et al.*, *Cancer Res.*, 54:3333-3336 (1994); Kerger, H. *et al.*, *Am. J. Physiol.*, 268:H802-H810 (1995)). Less than 1% O<sub>2</sub> corresponds to 6-7 torr. Hypoxic vessels were contracted with excess phenylephrine to maintain tone. SNO-Hb[FeII]O<sub>2</sub> (SNO-oxyHb) preparations were synthesized and 20 quantified as in Example 14; GSNO was prepared and assayed as described in Stamler, J.S. and Feelisch, M., "Preparation and Detection of S-Nitrosothiols," pp. 521-539 in *Methods In Nitric Oxide Research* (M. Feelisch and J.S. Stamler, eds.), John Wiley & Sons Ltd., 1996.

Hemoglobin is mainly in the R (oxy)-structure in both 95% O<sub>2</sub> or 21% O<sub>2</sub> (room 25 air) (M.F. Perutz, pp. 127-178 in *Molecular Basis of Blood Diseases*, G. Stamatyanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G. (John Wiley & Sons Inc., New York, 1995) pp. 215-235). Hb and SNO-Hb both contract blood vessels over this range of O<sub>2</sub> concentrations. That is, their hemes

sequester NO from the endothelium. The functional effects of these hemoproteins in bioassays are not readily distinguished (Figure 7A). Concentration-effect responses of SNO-Hb are virtually identical to those of native Hb in 95% O<sub>2</sub>--i.e., in R-structure (curves are not different by ANOVA; n=12 for each data point). Comparable contractile 5 effects were seen with up to 50 μM SNO-oxyHb/oxyHb--i.e., at doses where the responses had plateaued. Similar concentration-effect responses were observed in 21% O<sub>2</sub>, under which condition Hb/SNO-Hb is ~99% saturated (data not shown).

On the other hand, hypoxia (<1% O<sub>2</sub> [~6 mm Hg] simulating tissue PO<sub>2</sub>) which promotes the T-structure (M.F. Perutz, pp. 127-178 in *Molecular Basis of Blood 10 Diseases*, G. Stamatyanopoulos, Ed. (W.B. Saunders Co., Philadelphia, 1987); Voet, D. and Voet, J.G. (John Wiley & Sons Inc., New York, 1995) pp. 215-235), differentiates Hb and SNO-Hb activities: Hb strongly contracts blood vessels in T structure whereas SNO-Hb does not (Figure 7B). Concentration-effect responses of SNO-Hb and Hb are significantly different <1% O<sub>2</sub> (~6 torr), i.e. in T-structure. Native 15 deoxyHb is a powerful contractile agent whereas SNO-deoxyHb has a modest effect on baseline tone. (In most experiments SNO-Hb caused a small degree of contraction at lower doses and initiated relaxations at the highest dose; in some experiments (see Figure 8C) it caused dose-dependent relaxations.) n=13 for each data point; \*P<0.05; \*\*\*P<0.001 by ANOVA.

20 SNO-Hb relaxations are enhanced by glutathione through formation of S-nitrosoglutathione (GSNO) (Figure 7C). The potentiation of SNO-Hb vasorelaxation by glutathione is inversely related to the PO<sub>2</sub> (Figure 7C) because NO group transfer from SNO-Hb is promoted in the T-structure. Specifically, transnitrosation of glutathione by SNO-Hb--forming the vasodilator GSNO--is accelerated in T-structure (<1% O<sub>2</sub>). 25 Addition of 10 μM glutathione to bioassay chambers potentiates the vasorelaxant response of SNO-Hb. The potentiation is greatest under hypoxic conditions; i.e., the curve for <1% O<sub>2</sub> shows a statistically significant difference from both the 95% and 21% O<sub>2</sub> curves (P<0.001), which are not different from one another by ANOVA (n=6

for all data points). High concentrations of glutathione (100  $\mu$ M-1 mM) further potentiate SNO-Hb relaxations, such that the response is virtually identical to that seen in the presence of GSNO in Figure 7D. Glutathione at 10  $\mu$ M has no effect on native Hb contractions.

5 In contrast, the vasorelaxant effects of S-nitrosoglutathione are largely independent of  $PO_2$  (Figure 7D) and unmodified by superoxide dismutase (not shown). Concentration-effect responses of S-nitrosoglutathione (GSNO) are largely independent of  $PO_2$  in the physiological range (n=6 at each data point). Results are consistent with known resistance of GSNO to  $O_2/O_2^-$  inactivation (Gaston, B. *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:10957-10961 (1993)). Thus, in T-structure, relaxation by SNO overwhelms the contraction caused by NO scavenging at the heme, whereas the opposite is true in R-structure.

Example 10: Bioactivity of Intraerythrocytic S-Nitrosohemoglobin (SNO-RBCs)

Contractile effects of RBCs are reversed by intracellular SNO-Hb in low but not 15 high  $PO_2$ -i.e., under conditions that promote the T-structure. Low and high dose effects of SNO-RBCs are shown in Figures 8A and 8B, respectively.

Preparation of vessel rings and methods of bioassay are described in Example 9. SNO-oxyHb was synthesized and quantified as described in Example 14. Red blood 20 cells containing SNO-Hb (SNO-RBCs) were synthesized by treatment with tenfold excess S-nitrosocysteine over hemoglobin for 5-10 min. Under this condition, red blood cells are bright red and contain SNO-oxyHb; metHb was not detectable in these experiments.

Red blood cells containing SNO-Hb (SNO-RBCs) function in vessel ring 25 bioassays like cell-free SNO-Hb. In particular, low concentrations of SNO-RBCs (~0.1  $\mu$ M SNO-Hb) elicited modest contractile effects in 95%  $O_2$ , but not under hypoxia (Figure 8A). In 95%  $O_2$ , both SNO-RBCs (~0.1  $\mu$ M SNO-Hb[FeII] $O_2$ ) and native RBCs produced modest contractile effects that were not readily distinguished. The

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contractions by RBCs tended to be greater under hypoxic conditions (<1% O<sub>2</sub>), whereas those of SNO-RBCs were reversed (slight relaxant effects were seen). These O<sub>2</sub>-dependent responses of SNO-RBCs closely resemble those of cell-free preparations. Hemolysis was minor and could not account for the observed effects.

5 At higher concentrations, SNO-RBCs produced small transient relaxations in 95% O<sub>2</sub> and larger sustained relaxations under hypoxia (Figure 8B), much like cell-free SNO-Hb in the presence of glutathione. For example, SNO-RBCs (~1 μM SNO-Hb[FeII]O<sub>2</sub>) caused 32.5±1.2% relaxation that lasted 14.5±0.7 min. in 95% O<sub>2</sub> versus 61±10% relaxation that lasted 23± min. in <1% O<sub>2</sub> (n=3-4; P<0.05). In contrast, RBCs  
10 containing no SNO-Hb produced small contractions (less than those of cell-free Hb) that are potentiated by hypoxia (13±2.0% in 95% O<sub>2</sub> vs. 25±5% in <1% O<sub>2</sub>; P<0.05). Hemolysis in these experiments was minor and could not account for the extent of relaxation by SNO-RBCs.

In 95% O<sub>2</sub>, SNO-RBCs (~1 μM SNO-Hb[FeII]O<sub>2</sub>) produced relaxations of aortic  
15 rings, whereas native RBCs produced slight contractions. Both effects were more prominent at low PO<sub>2</sub>. That is, relaxations and contractions of intraerythrocytic SNO-Hb and Hb, respectively, were greater and longer-lived in <1% O<sub>2</sub> than in 95% O<sub>2</sub>. The O<sub>2</sub>-dependent responses of SNO-RBCs mimicked those of cell-free SNO-Hb in the presence of glutathione. Hemolysis in these experiments was minor and could not  
20 account for the extent of relaxation by SNO-RBCs.

The normal response of systemic arteries to hypoxia is dilation and contraction to high PO<sub>2</sub>. The responses of vessel rings to changes in PO<sub>2</sub> in the presence of SNO-Hb and Hb were tested (Figure 8C). Vessel rings were contracted with phenylephrine under hypoxic conditions (6-7 torr) and then exposed to either 1 μM Hb or SNO-Hb.  
25 Hb produced progressive increases in vessel tone, while SNO-Hb caused relaxations. Introduction of 95% O<sub>2</sub> led to rapid contractions in both cases. Thus, structural changes in SNO-Hb effected by PO<sub>2</sub> are rapidly translated into contractions or relaxations, whereas Hb contracts vessels in both R- and T-structures. Thus, Hb opposes the

physiological response and SNO-Hb promotes it (Figure 8C). Direct effects of O<sub>2</sub> on smooth muscle operate in concert with SNO-Hb to regulate vessel tone.

Example 11: Influence of O<sub>2</sub> Tension on Endogenous Levels of S-Nitrosohemoglobin (SNO/Hb) and Nitrosyl Hemoglobin (FeNO/Hb)

5 Allosteric control of SNO-Hb by O<sub>2</sub> was assessed *in vivo* by perturbation of the periarteriolar oxygen gradient. In animals breathing room air (21% O<sub>2</sub>), the precapillary resistance vessels (100-10  $\mu\text{m}$ ) are exposed to PO<sub>2</sub>s as low as 10-20 torr (Duling, B. and Berne, R.M. *Circulation Research*, 27:669 (1970); Popel, A.S., *et al.*, (erratum *Am. J. Physiol.* 26(3) pt. 2) *Am. J. Physiol.* 256, H921 (1989); Swain, D.P. and Pittman, R.N., 10 *Am. J. Physiol.* 256, H247-H255 (1989); Buerk, D. *et al.*, *Microvasc. Res.*, 45:134-148 (1993)) (confirmed here) which promotes the T-structure in Hb. Raising the inspired oxygen concentration to 100% translates to periarteriolar PO<sub>2</sub>s only as high as 40 mm Hg (Duling, B. and Berne, R.M. *Circulation Research*, 27:669 (1970); Popel, A.S., *et al.*, (erratum *Am. J. Physiol.* 26(3) pt. 2). *Am. J. Physiol.* 256, H921 (1989); Swain, D.P. 15 and Pittman, R.N. *Am. J. Physiol.* 256, H247-H255 (1989); Buerk, D. *et al.*, *Microvasc. Res.*, 45:134-148 (1993)); i.e., breathing 100% O<sub>2</sub> may not fully maintain the R-structure in Hb in the microcirculation. Elimination of the periarteriolar O<sub>2</sub> gradient (artery-arteriole and arterial-venous difference in PO<sub>2</sub>) is accomplished in hyperbaric chambers by applying 3 atmospheres of absolute pressure (ATA) while breathing 100% 20 O<sub>2</sub> (Tibbles, P.M. and Edelsberg, J.S., *N.E.J.M.*, 334:1642-1648 (1996)).

Adult male Sprague-Dawley rats (290-350g) were anesthetized with sodium pentobarbital (50 mg/kg IP), intubated and ventilated with a small animal respirator (Edco Scientific Inc., Chapel Hill, NC) at a rate and tidal volume to maintain normal values of PaCO<sub>2</sub> (35-45 mm Hg; PaCO<sub>2</sub> = systemic arterial blood carbon dioxide tension). The femoral vein and artery were cannulated for infusion of drugs and for continuous monitoring of systemic blood pressure, respectively. Aliquots of arterial blood (200  $\mu\text{l}$ ) were drawn periodically to measure blood gas tensions and pH 25

(Instrumentation Laboratory Co., model 1304 blood gas/pH analyzer). The blood was replaced intravenously with three volumes of normal saline. The inspired O<sub>2</sub> concentration was varied using premixed gases balanced with nitrogen. The tissue PO<sub>2</sub> was measured continuously with polarographic platinum microelectrodes (50 µm O.D. 5 coated with hydrophobic gas permeable Nafion) implanted stereotactically in both the right and left hippocampus (AP-3.4 mm, ML+2.2 mm), caudate putamen nucleus and substantia nigra (see coordinates below) (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981); Dewhirst, M.W. et al., *Cancer Res.*, 54:3333-3336 (1994); Kerger, H. et al., *Am. J. Physiol.*, 268:H802-H810 (1995)). The 10 PO<sub>2</sub> electrodes were polarized to -0.65V against a distant Ag/AgCl reference located on the tail and the current flow was measured using a low-impedance nA-meter. Regional arterial PO<sub>2</sub> was adjusted by changing the inspired O<sub>2</sub> concentration and atmospheric pressure.

Polarographic hydrogen (H<sub>2</sub>)-sensitive microelectrodes were implanted 15 stereotactically in the substantia nigra (AP -5.3 mm, ML -2.4 mm to the bregma, depth 3.2 mm), caudate putamen nucleus (CPN) (AP +0.8 mm, ML -2.5 mm, depth 5.2 mm) and parietal cortex, for measurement of regional blood flow (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981)). The microelectrodes were made from platinum wire and insulated with epoxy, with the 20 exception of the tip (1 mm) which was coated with Nafion. For placement, the electrodes were mounted on a micromanipulator and the rat's head was immobilized in a Kopf stereotaxic frame. H<sub>2</sub>-sensitive electrodes were polarized to +400 mV against a distant reference electrode on the tail, and the polarographic current was measured using a low-impedance nA meter during and after the inhalation of hydrogen gas (2.5%) for 1 25 min. Both the hydrogen clearance curves and voltage for oxygen measurements were made using PC WINDAQ (software, DI-200 AC, Dataq Instruments, Inc., Akron, OH). Cerebral blood flow was calculated using the initial slope method (Young, W., *Stroke*, 11:552-564 (1980); Heiss, W.D. and Traupett, H., *Stroke*, 12:161-167 (1981)).

Regional blood flow responses were monitored for 30 min. prior to and 30 min. following drug administration; hemoglobins were given at time 0.

Blood was drawn from indwelling catheters in the carotid artery (arterial blood that perfuses the brain) and superior vena cava/right atrium (venous return to the heart)

5 of 5 rats exposed first to room air (21% O<sub>2</sub>) and then 100% O<sub>2</sub> + 3 ATA in a hyperbaric chamber. Levels of SNO-Hb and nitrosyl Hb (Hb[Fe]NO; FeNO in Figure 9) were determined from these samples (by methods in Example 8) as a measure of SNO-Hb and nitrosyl Hb (Hb[Fe]NO) in blood that perfuses the brain. The mean O<sub>2</sub> saturation of venous blood (room air) was 69%; of arterial blood (room air) was 93%; of venous

10 blood (100% + 3 ATA) was also 93% and of arterial blood (100% + 3 ATA) was 100% (Figure 9). Numerous statistical comparisons were highly significant. For example, SNO-Hb venous 100% O<sub>2</sub> + 3 ATA vs. SNO-Hb venous 21% O<sub>2</sub>, P=0.004; and nitrosyl Hb venous 21% O<sub>2</sub> vs. arterial 21% O<sub>2</sub> P=0.008. On the other hand, SNO-Hb and nitrosyl Hb were not different in artery 21% O<sub>2</sub> compared with venous 100% + 3 ATA

15 (which have identical O<sub>2</sub> saturations), nor did the differences reach significance between venous and arterial 100% O<sub>2</sub> + 3 ATA. n=5 for all measurements.

In 21% O<sub>2</sub>, venous blood contained mostly nitrosyl Hb, whereas arterial blood contained significant amounts of SNO-Hb (Figure 9). On the other hand, SNO-Hb predominated in both arterial and venous blood in 100% O<sub>2</sub> + 3 ATA (Figure 9). In

20 hyperbaric conditions, the tissues are oxygenated primarily by O<sub>2</sub> dissolved in plasma. Physiologically circumventing the unloading of O<sub>2</sub> by Hb alters the endogenous SNO/nitrosyl Hb balance. The data show that SNO-Hb appears to form endogenously in R-structure whereas SNO is released in the T-structure (compare venous 21% O<sub>2</sub> (T-state) with arterial 100% O<sub>2</sub> + 3 ATA (R-state)).

25 This structure-function relationship *in vivo* is consistent with both the *in vitro* pharmacology and the molecular model suggesting that 1) O<sub>2</sub> is an allosteric effector of Hb S-nitrosylation; 2) binding of NO to hemes of Hb is favored in the T-structure; (some of the NO released during arterial-venous (A-V) transit appears to be

autocaptured at the hemes) and 3) maintaining endogenous SNO-Hb in the R-structure by eliminating the A-V O<sub>2</sub> gradient preserves levels of SNO (compare venous 100% O<sub>2</sub> + 3 ATA with arterial 21% O<sub>2</sub>). Thus, it can be predicted that SNO-Hb should improve cerebral blood flow in 21% O<sub>2</sub>, under which condition SNO is readily released during 5 A-V transit, but not under the hyperoxic conditions that maintain the R-structure in artery and vein.

Example 12: O<sub>2</sub>-Dependent Effects of SNO-Hb and Hb on Local Cerebral Blood Flow

The cerebrovascular effects of SNO-Hb were measured in adult male Sprague-Dawley rats using O<sub>2</sub> and H<sub>2</sub> (blood flow)-sensitive microelectrodes that were placed 10 stereotactically in several regions of the brain as for Example 11.

SNO-Hb increases blood flow under tissue hypoxia, whereas it decreases blood flow under hyperoxia. In contrast, Hb decreases blood flow irrespective of the PO<sub>2</sub>. Comparative effects of SNO-Hb (●) and Hb (■) (1 μmol/kg infused over 3 minutes) on local blood flow in substantia nigra (SN), caudate putamen nucleus, and parietal cortex 15 are shown for three different conditions. In 21% O<sub>2</sub>, SNO-Hb improved blood flow in all three regions of the brain tested, whereas native Hb decreased local blood flow, paradoxically attenuating O<sub>2</sub> delivery to hypoxic tissues (Figures 10A, 10B and 10C; all curves are highly statistically significantly different from one another and from baseline by ANOVA). In rats breathing 100% O<sub>2</sub>, where the periarteriolar O<sub>2</sub> gradient has been 20 essentially eliminated, the increase in flow to SNO-Hb was significantly attenuated (i.e., only the SN increase reached statistical significance), but the Hb-mediated decrease in flow was preserved (Figures 10D, 10E and 10F; all curves remain different from one another by ANOVA to P<0.05). In 100% O<sub>2</sub> + 3 ATA, both SNO-Hb and Hb tended to decrease cerebral flow to similar extents (Figures 10G, 10H and 10I; curves are not 25 different by ANOVA). S-nitrosoglutathione (GSNO) increased brain perfusion in 100% O<sub>2</sub> and 100% O<sub>2</sub> + 3 ATA, reversing protective vasoconstriction (not shown). Baseline blood flow was decreased by ~10% under 100% O<sub>2</sub> + 3 ATA as compared to 100% O<sub>2</sub>.

n=7 for all data points. Values of tissue/microvascular PO<sub>2</sub> ranged from 19-37 mm Hg in 21% O<sub>2</sub>; from 68-138 mm Hg in 100% O<sub>2</sub>; and from 365-538 mm Hg in 100% + 3 ATA (Duke University Medical Center Hyperbaric Chambers).

SNO-Hb acts like native Hb (net NO scavenger) when it is in the R (oxy)-  
5 structure and like GSNO (net NO donor) in the T (deoxy)-structure. The results are consistent with the conclusion that SNO-Hb is a nitrosothiol whose vasoactivity is allosterically controlled by PO<sub>2</sub>.

Example 13: Hemodynamics of Cell Free and Intraerythrocytic SNO-Hb, Hb and GSNO at Different O<sub>2</sub> Concentrations

10 Rats were anesthetized by intraperitoneal injection of pentobarbital, and the femoral arteries and veins accessed by local cutdown. The artery was then cannulated and the blood pressure monitored continuously using a P23 XL pressure transducer (Viggo Spectramed, Oxnard, CA) attached to a Gould recorder. The femoral vein was used for infusion of drugs and red blood cells containing SNO-Hb (1 ml over 1 min.)  
15 and an IBM PC (WINDAQ 200, Dataq Instruments, Inc.; Akron, OH) was used for data acquisition.

Drugs were infused through the femoral vein at 1  $\mu\text{mol}/\text{kg}$  infused over 1 minute after blood pressure had stabilized (approximately 30 min). Measurements shown (Figure 11A) were taken at 10 min. post-infusion of drug. Similar responses were seen  
20 at 3 and 20 min. SNO-Hb produced significantly less of an increase in blood pressure than Hb ( $P<0.05$ ), whereas GSNO decreased blood pressure.  $P<0.05$  vs. SNO-Hb;  
 $*P<0.05$ ,  $**P<0.01$ ) vs. baseline blood pressure. n=5-6 for each drug.

Infusions of SNO-RBCs also lowered blood pressure consistent with a GSNO-like effect (Figure 11B). SNO-RBCs produced dose-dependent hypotensive effects  
25 (similar to those of cell-free SNO-Hb) ( $P<0.001$  at all points vs. baseline). The hypotensive effects of SNO-RBCs were potentiated by pre-administration of the NO synthase inhibitor N<sup>G</sup>-monomethyl-L-arginine (L-NMMA; 50 mg/kg). n=8 for each

data point. Curves different by ANOVA ( $P<0.01$ ), \* $P<0.05$  vs. L-NMMA. The amount of hemolysis in these experiments was trivial. Infusion of the hemolysate had no effect on blood pressure.

NO synthase inhibition increases tissue  $O_2$  consumption by relieving the  
5 inhibition of mitochondrial respiration produced by NO in the tissues (King, C.E. *et al.*,  
*J. Appl. Physiol.*, 76(3):1166-1171 (1994); Shen, W. *et al.*, *Circulation*, 92:3505-3512  
(1995); Kobzik, L. *et al.*, *Biochem. Biophys. Res. Comm.*, 211(2):375-381 (1995)). This  
should, in turn, increase the periarteriolar  $O_2$  gradient which might explain some of the  
10 potentiation. However, other factors, such as a change in tone or distribution of blood  
flow imposed by L-NMMA, may well contribute. The effects of SNO-Hb on blood  
pressure are consistent with SNO being released in resistance arterioles to compensate  
for NO scavenging at the heme iron.

Example 14: Synthesis of S-Nitroso-Oxyhemoglobin (SNO-Hb[Fe(II)]O<sub>2</sub>) and SNO-metHb (SNO-Hb[FeIII])

15 Hemoglobin (Hb)A<sub>o</sub> was purified from human red blood cells as previously  
described (Kilbourn, R.G. *et al.*, *Biochem. Biophys. Res. Commun.*, 199:155-162  
(1994)). HbA<sub>o</sub> was dialyzed against 2% borate, 0.5 mM EDTA (pH 9.2) at 4°C for 12-  
16 hours. The oxyHb concentration was determined based on the optical absorbance at  
577 nm (i.e., using the millimolar extinction coefficient 14.6).

20 Hb was reacted with 10-fold molar excess S-nitrosocysteine (CYSNO) which  
was synthesized with modification of standard procedure (see, for example, Stampler,  
J.S. and Feelisch, M., "Preparation and Detection of S-Nitrosothiols," pp. 521-539 in  
*Methods In Nitric Oxide Research* (M. Feelisch and J.S. Stampler, eds.), John Wiley &  
Sons Ltd., 1996) as follows. L-cysteine hydrochloride (1.1 M) dissolved in 0.5 N  
25 HCl/0.5 mM EDTA was reacted with an equal volume of 1 M NaNO<sub>2</sub> (sodium nitrite)  
dissolved in water, to form CYSNO (the ratio of cysteine to nitrite influences the SNO-  
Hb product and activity profile). The concentration of CYSNO was then adjusted by

dilution in 200 mM phosphate buffer (PBS), pH 8.0, to yield a working CYSNO solution (pH 6-7). This was then reacted at room temperature with a 20-fold volume excess of Hb (in borate, pH 9.2), resulting in a final 10-fold molar excess of CYSNO over Hb (ratio influences product critically). Following the incubation (periods  
5 determined by the desired synthetic preparation; i.e., a desired ratio of SNO/tetramer; desired met- to oxy- to nitrosyl-Hb ratios; polynitrosated or non-polynitrosated; for example, 10 min. preferred time for SNO-oxyHb with 2 SNO per tetramer; see below), the reaction mixture was rapidly added to a column of fine Sephadex G-25 (20 to 30-fold volume excess over the reaction mixture) preequilibrated with 100 mM PBS pH  
10 7.4, 0.5 mM EDTA. Typically, a 150 µl sample of the mixture was added to a 4.5 ml column measuring 12 mm (inner diameter). The column was then centrifuged at 1200 g for 60 seconds and the effluent collected in a 1.5 ml airtight plastic vial that was subsequently kept on ice and protected from light.

Spectrophotometric determination of total Hb and S-nitrosothiol (SNO)  
15 concentration was determined by Saville assay (especially modified from standard protocol (see Materials and Methods section of Examples; see also, for example, Stamler, J.S. and Feelisch, M., "Preparation and Detection of S-Nitrosothiols," pp. 521-539 in *Methods In Nitric Oxide Research* (M. Feelisch and J.S. Stamler, eds.) John Wiley & Sons Ltd., 1996; Saville, B., *Analyst*, 83:670-672 (1958)) by keeping the Hb in  
20 the sample to 5 µl per 50 µl sample volume added to assay, adding 0.1-1% Tween as necessary, and by correcting for nitrosyl Hb content converted to nitrite).

Incubations of CYSNO with HbA resulted in different synthetic products (and different activities) over time. For example, with 10 min incubations, the Hb preparation contains  $1.857 \pm 0.058$  SNO groups per tetramer, and is approximately 12-25 15% metHb and 1-3% nitrosyl(FeII)-hemoglobin. (Capillary electrophoretic analysis actually reveals a mixture of three protein peaks.) MetHb was then reduced (lowered from 13% to 2% with 100-fold excess NaCNBH<sub>3</sub> (dissolved in PBS, pH 8.0) under anaerobic conditions (achieved by purging with argon gas for a minimum of 10

minutes) for 5 minutes. (Lower concentrations of NaCNBH<sub>3</sub> or treatment of the samples under aerobic conditions were not effective in lowering the metHb concentration, and alternative measures to reduce the heme result in SNO reduction.) The resulting mixture was rapidly added to a column of fine Sephadex G-25 (20 to 30-fold volume excess) preequilibrated with 100 mM PBS pH 7.4, 0.5 mM EDTA. The final S-nitrosothiol/Hb tetramer ratios were not significantly different from those measured in samples degassed and treated with PBS only: losses in SNO/Hb ratio relative to the starting ratios were consistent with the expected time-dependent decay of SNO-deoxyHb and could be reduced to an insignificant loss by taking preparative time into consideration. NaCNBH<sub>3</sub> treatment of a sample with a mean SNO/Hb ratio of ~1 decreased the metHb content from 5.6% to 0.63%. Nitrosyl(FeII)-hemoglobin and metHb contamination of SNO-oxyHb preparations of ~2% are acceptable, inasmuch as they do not seem to alter bioactivity of SNO-oxyHb, and enable O<sub>2</sub> binding measurements, that is, P<sub>50</sub> determinations (so called SNO-Hb[Fe(II)]O<sub>2</sub>). By the same token, bioactivity can be modified and varied by controlling the proportion of SNO-metHb (high and low spin) and nitrosyl(FeII)-hemoglobin in the preparation. The spin state of metHb can be controlled by the heme ligand: cyan-metHb is low spin and aquo-met Hb (H<sub>2</sub>O bound as ligand) is high spin; nitrosyl Hb ratios are made depending on the desired result. High yield SNO-metHb, SNO-nitrosyl(FeII)-hemoglobin (or SNO-carboxyl Hb) can be formed by using the heme-liganded protein as starting material. Carboxyl Hb can be made by gassing Hb with CO under anaerobic conditions. HbCO can then be used as starting material; likewise, various combinations of Hb[FeNO][FeCO] can be used as starting material.

Example 15: Measurements of NO as SNO and Iron Nitrosylhemoglobin in Blood of  
25 PPH Patients and Normal Subjects

Levels of total hemoglobin, iron nitrosylhemoglobin, and SNO-hemoglobin within red blood cells were analyzed in blood drawn from normal human subjects and

patients with primary pulmonary hypertension. Concentrations of hemoglobin were determined by spectrophotometric measurements. Concentrations of iron nitrosylhemoglobin and SNO-hemoglobin were determined by photolysis/chemoluminescence analysis (US 5,891,735). Shown in Figures 12A, 12B and 12C are the individual values and the mean for (Figure 12A) S-nitrosothiol, (Figure 12B) iron nitrosylhemoglobin, and (Figure 12C) total NO.

Mean NO bound to hemoglobin in patients with PPH is significantly lower than in normal controls.

#### Example 16: Loss of SNO From Stored Blood

10 Freshly drawn human red blood cells were stored for up to 2 hours at room temperature. At the stated intervals, the red blood cells were subjected to analysis of S-nitrosothiol content following oxygenation, using standard chemiluminescent techniques (US 5,891,735; also see Example 8). A substantial loss of S-nitrosothiol is seen over the 2-hour period. See Figure 13.

15 Freshly drawn human blood and human blood that had been frozen at -80°C for 4 days were analyzed for NO content as S-nitrosothiol and as iron nitrosylhemoglobin. Figure 14 shows that blood stored for 4 days, even if frozen, suffered a dramatic loss in S-nitrosothiol. NO moves from thiols to hemes (of the  $\alpha$  chains, as EPR shows hyperfine structure over time -- data not shown), and does not go back to thiols. Stored  
20 blood also loses ATP.

#### Example 17: Effects of Ethyl Nitrite on Red Blood Cell NO

A patient with primary pulmonary hypertension, with blood having starting values for SNO-hemoglobin, iron nitrosylhemoglobin and total NO/hemoglobin molar ratio as shown in the left bar of Figure 15, was given an NO-donating drug by inhalation. Ethyl nitrite was diluted in ethanol. Oxygen carrier gas was passed through the ethyl nitrite solution and delivered to the patient at 10 liters/min. After 10 minutes,

blood was withdrawn and analyzed by photolysis chemoluminescence. Results are shown in the right bar of Figure 15, for the patient's blood after treatment. SNO-Hb and total Hb-bound NO levels were depressed in the patient relative to normal controls, but rose during inhalation of ethyl nitrite gas. The experiment was repeated for other 5 patients (n=5). The increase in SNO-Hb upon ethyl nitrite treatment was statistically significant (p < 0.05). There was no statistical difference in the levels of iron nitrosylhemoglobin before and after treatment

Further evidence of the increase in NO bioactivity due to ethyl nitrite is shown in Figure 16. These changes paralleled decreases in pulmonary vascular resistance and 10 improved systemic oxygenation.

#### Example 18: Treatment of RBCs with NO Increases Coronary Blood Flow

Mongrel dogs (25-30 kg) were anesthetized with thiamylal sodium (60-80 mg/kg). A thoracotomy was performed in the left fourth intercostal space and the heart suspended in a pericardial cradle. Heparin-filled catheters were placed in the left 15 ventricular cavity, left atrium and ascending aorta. The left circumflex coronary artery (CCA) distal to the left atrial appendage was dissected free. 7 MHz piezoelectric crystals were sutured on opposite sides of the CCA. A pulse Doppler flow probe (19 MHz, cuff-type) was implanted distal to the crystals and an inflatable balloon occluder was placed distal to the flow probe. All arterial branches between the crystals and the 20 occluder were ligated. All catheters, tubing and wires were tunneled to a subcutaneous pouch at the base of the neck. After a 10-15 day recovery period, the catheters and wires were exteriorized under general anesthesia. Two to three days later, a bolus injection of nitroglycerin (GTN, 0.4 mg) was administered to confirm vascular responsiveness. Animals demonstrating <5% increase in diameter of the epicardial 25 vessels were excluded from subsequent study.

Human red blood cells in whole blood from normal human were loaded using a solution of NO (1:250 NO to hemoglobin ratio, producing iron nitrosylhemoglobin)

under deoxygenated conditions, and the red blood cells were rapidly oxygenated (thereby converting iron nitrosylhemoglobin to SNO-Hb; see Gow, A.J. and Stamler, J.S., *Nature* 391:169-173, 1998). 1-100 ml of the blood were infused into a dog. Coronary flow, left ventricular end diastolic pressure (LVEDP) and blood pressure were measured before and after the infusion. LVEDP was measured with a catheter placed in the left ventricle. Blood pressure was measured via an arterial line. The percent of change for each of these parameters is shown in Figure 17. Coronary blood flow increased, while blood pressure was reduced.

## Example 19: Red Blood Cells Treated to Increase SNO Reduce Pulmonary Artery Pressure

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise specified. These experiments were performed in isolated buffer-perfused lungs (IPL) of rabbits as previously described (Nozik-Grayck, E., et al., *American Journal of Physiology* 273:L296-304, 1997). The buffer was Krebs-Henseleit  
15 (KH) solution containing sodium chloride (82.9 mM), potassium chloride (4.7 mM), monobasic potassium phosphate (2.4 mM), sodium bicarbonate (25 mM), magnesium sulfate (1.2 mM), calcium chloride (2.7 mM), and dextrose (11.1 mM) at pH 7.4. New Zealand white rabbits (May's Farm, NC) weighing 2.5-3.5 kg were anticoagulated with 5,000 U sodium heparin and anesthetized with 25 mg/kg sodium pentobarbital by ear  
20 vein. An incision was made in the left chest wall, exposing the heart. The animal was exsanguinated via the left ventricle and the thorax entered by excising the rib cage. Stainless steel cannulas were placed in the trachea, main pulmonary artery and left atrium. The aorta also was tied with the pulmonary artery to prevent loss of perfusate to systemic circulation. The lungs were inflated with 80 ml of air and ventilated with 21%  
25 O<sub>2</sub> and 5% CO<sub>2</sub> (balance N<sub>2</sub>) with an animal respirator (Harvard Apparatus Company, Inc., S. Natick, MA) at a rate of 30 breaths/minute. The tidal volume was adjusted to

maintain a peak tracheal pressure of 8-10 torr with a positive end expiratory pressure of 2-3 torr.

The perfusion circuit contained a reservoir suspended freely from a force transducer (Model FT100, Grass Instrument Company, Quincy, MA) and a water heater set at 37°C. Perfusate was circulated by a roller pump (Sarns, Inc., Ann Arbor, MI) and passed through a bubble trap before entering the pulmonary artery. The perfusate returned to the left atrium and then to the reservoir which was set at the lowest portion of the lung to provide a left atrial pressure of zero. Perfusion began slowly and was gradually increased to 100 ml/min. After rinsing the lungs free of blood with 500 ml buffer, a recirculating system was established. The total volume in the circuit was approximately 250 ml. Mean pulmonary artery pressure (PA) and tracheal pressure (Paw) were measured using pressure transducers (P231D, Gould Stratham Instruments, Inc., Hato Ray, PR). The weight gain (WG) of the lung as an index of pulmonary edema formation was measured as the loss of perfusate from the reservoir connected to the force transducer. PA, Paw and WG were continuously recorded on a four channel recorder (model 2450S, Gould Inc., Cleveland, OH). The preparation was considered successful if the PA was stable between 10 and 20 torr and there was less than 0.15 g/min weight gain during a ten minute stabilization period.

Human red blood cells were treated to increase intracellular S-nitrosothiol. In one case, NO was added to deoxygenated red blood cells at 1:250 NO:Hb as described in Pawloski *et al.*, *Nature* 409:622-626, 2001. In an alternative method, S-nitrosocysteine was incubated with the red blood cells for 10 minutes at a CYSNO:Hb ratio of 1:250.

Measurements in pulmonary artery pressure across whole lung were made before and after the addition to the perfusate of 0.1 ml treated red blood cells. The percent change in pulmonary artery pressure is shown in Figure 18.

Perfusing the lungs with untreated red blood cells causes a pulmonary hypertensive effect, which worsens ventilation perfusion mismatching. Both red blood

cells treated with NO (NO-RBCs; initially forming iron nitrosylhemoglobin, followed by endogenous formation of SNO-Hb), and red blood cells treated with CYSNO (SNO-RBC), when perfused into isolated rabbit lung, have the opposite effect. See Figure 18.

Example 20: Vasoactivity of Sickle Cell Patient Red Blood Cells

5       The experimental procedure was as described in Pawloski, J.R. *et al.*, *Nature* 409:622-626 (2001) and in Stamler, J.S. *et al.*, *Proc. Natl. Acad. Sci. USA* 89:444 (1992). Rabbit aortic rings were suspended in Krebs-bicarbonate buffer at 37°C, bubbled continuously with either 95% O<sub>2</sub>/5% CO<sub>2</sub> or 95% argon/5% CO<sub>2</sub> (measured O<sub>2</sub> less than 1%). Resting tension was maintained at a standard 2 g and active tension was  
10 induced by phenylephrine. NO-treated and control red blood cells were washed and resuspended in PBS at 50% hematocrit, and added to individual 25-ml baths as 0.2-ml aliquots to yield a bath hematocrit of 0.4%.

Untreated red blood cells from normal humans dilated blood vessels, while untreated red blood cells from sickle cell patients caused a contraction of blood vessels.  
15 Red blood cells from sickle cell patients, treated with NO at a NO:hemoglobin ratio of 1:250, caused the dilation of blood vessels to some extent, but not as much as normal red blood cells. The extent of dilation was inversely proportional to the severity of the clinical disease. See Figure 19.

Example 21: Increase in Coronary Blood Flow From NO-Loaded Red Blood Cells Is  
20 Inversely Proportional to pO<sub>2</sub>

Red blood cells were loaded with NO (at a 1:25 NO to hemoglobin ratio) under deoxygenated conditions, and then rapidly oxygenated. One hour later the red blood cells were infused into the circumflex coronary artery of instrumented dogs and coronary blood flow was measured by Doppler (as described above in Example 18).  
25 Transfusions varied but were designed to effectively keep NO:hemoglobin ratios in the 1:1000-1:25 range (independent of hematocrit).

Shown in Figures 20A and 20B are plots of blood flow in the left circumflex coronary artery, under normoxia (95% O<sub>2</sub>; Figure 20A) or ischemia (60% O<sub>2</sub>; Figure 20B). It can be seen that loading red blood cells with NO increases coronary flow, from dilation of the coronary blood vessels. Flow-mediated increases are proportional to 5 ischemia, and inversely proportional to pO<sub>2</sub>. The changes in coronary blood flow in response to the transfusion of untreated (native) RBCs or NO-loaded RBCs were plotted for both the normoxic and hypoxic conditions. See Figure 21.

Example 22: CysNOEE Increases RBC Membrane SNO and NO Bioactivity of RBCs

S-nitrosocysteine ethyl ester (CysNOEE) was added to human whole blood to 10 interact with the red blood cells. CysNOEE:Heme (see Figure 22) is the molar ratio of CysNOEE to heme in hemoglobin contained in red blood cells, for CysNOEE incubated with blood for 5 minutes at room temperature. After these incubations at different CysNOEE:heme ratios (1:100, 1:10, 1:1 and 10:1; see Figure 22), red blood cell membranes were isolated by lysis and centrifugation, and S-nitrosothiol (SNO) content 15 of the isolated membranes was determined by photolysis chemiluminescence assay. The concentrations of SNO in the red blood cell membranes were plotted in Figure 22 as membrane SNO. Bioactivity of red blood cells -- from release of biologically active NO -- was determined in a rabbit aortic ring bioassay under two different concentrations of oxygen, and the results are plotted in Figure 22 as 21% oxygen, vasoactivity, and 1% 20 oxygen, vasoactivity. S-nitrosocysteine ethyl ester relaxes blood vessels, most effectively under ischemic conditions.

Example 23: Glutathione Potentiates Bioactivity of RBCs

NO-loaded red blood cells were produced by incubating whole human blood in a solution comprising S-nitrosocysteine (CYSNO) at a molar ratio of 1:100 25 CYSNO:hemoglobin for 5 minutes at room temperature. The bioactivity of the treated blood was tested in a rabbit aortic ring bioassay, in the presence (+GSH) or absence

(-GSH) of 100  $\mu\text{M}$  glutathione. The results are given in Table 3. Glutathione potentiates the bioactivity of red blood cell-mediated vasodilation.

Table 3

	% Change in Tone	
	CysNO-RBC (1:100)	
	95% Argon	
	<u>(+GSH)</u>	<u>(-GSH)</u>
5	-40	-29
	-45	-17
10		-19
		-21
		-36
		-17
15	Mean -43	-23
	SEM 0.8	0.3

#### Equivalents

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.